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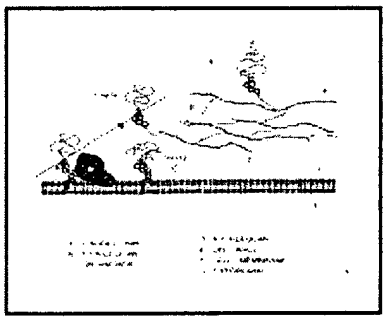
(54) **Title:** METHOD OF SCREENING COMPOUND HAVING FUNGAL CELL WALL SYNTHESIS INHIBITORY ACTIVITY

(54) 発明の名称: 真菌細胞壁合成阻害活性を有する化合物をスクリーニングする方法

(57) Abstract

By a simple binding assay with the use of a membrane fraction in which GWT1 protein is expressed, a compound inhibiting the transport of GPI anchor protein to fungal cell wall can be screened.

(57) 要約



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METHOD OF SCREENING COMPOUND HAVING FUNGAL CELL WALL SYNTHESIS INHIBITORY ACTIVITY
[SHINKIN SAIBOUHEKI GOSEI SOGAI KASSEI WO YUSURU KAGOBUTSU WO SUKURININGU SURU HOHO]

TSUKAHARA et al

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TITLE (54): METHOD OF SCREENING COMPOUND HAVING FUNGAL CELL WALL SYNTHESIS INHIBITORY ACTIVITY

FOREIGN TITLE [54A]: SHINKIN SAIBOUHEKI GOSEI SOGAI KASSEI WO YUSURU KAGOBUTSU WO SUKURININGU SURU HOHO

Specification

Screening method for compound having fungal cell wall synthesis inhibitory activity

Field of the Invention

The present invention relates to screening methods for antifungal agents which bind to proteins that are involved in the synthesis of fungal cell walls.

Background of the Invention

The present inventors noticed that adhesion to host cells is important to enable fungi to exert their pathogenicity, and that adhesion factors involved in fungal cell adhesion are transported to the surface layers of cell walls after glycosylphosphatidylinositol (GPI) anchors on the cell membrane (Hamada K et al., Mol. Gen. Genet., 258: 53-59, 1998). Accordingly, the present inventors considered that novel antifungal agents that inhibited the synthesis of fungal cell walls and also inhibited the adhesion of fungal cells to host cells could be generated by inhibiting the process of transporting proteins anchored with GPI (GPI-anchored proteins) to cell walls. Thus, the present inventors started to study the subject.

Disclosure of the Invention

The present invention intends to develop antifungal agents that prevent pathogenic fungi from exerting pathogenicity, by inhibiting the synthesis of fungal cell walls, as well as by inhibiting fungal cell adhesion to host cells, by inhibition of the transport of GPI-anchored proteins to fungal cell walls.

The present inventors found the following proteins involved in the process of transporting GPI-anchored proteins to cell walls: the proteins of Saccharomyces cerevisiae encoded by DNAs comprising the nucleotide sequence of SEQ ID NO: 1; the proteins of Candida albicans encoded by DNAs comprising the nucleotide sequences of SEQ ID NOs: 3 and 5; the proteins of Schizosaccharomyces pombe encoded by DNAs comprising the nucleotide sequence of.SEQ

ID NO: 27; the proteins of Aspergillus fumigatus encoded by DNAs comprising the nucleotide sequences of SEQ ID NOs: 39 and

/2

41; and the proteins of Cryptococcus neoformans encoded by DNAs comprising the nucleotide sequences of SEQ ID NOs: 54 and 58. These nucleotide sequences were named "GWT1 genes". Further, the inventors discovered that GWT1 gene-deficient fungi could not synthesize cell walls and the compound represented by formula (Ia) bound to the above-described proteins in order to inhibit the transport of GPI-anchored proteins to cell walls, resulting in the synthesis of fungal cell walls being inhibited.

The inventors further discovered that compounds which bind to the proteins against the compounds that can be represented by formula (Ia) could inhibit the synthesis of fungal cell walls. The present invention is based on these discoveries.

Specifically, the present invention provides the following items 1 to 7:

1. A screening method for a compound having an antifungal activity, wherein the method comprises the steps of:

(1) A process wherein a protein encoded by a DNA described in any of items (a) to (e),, a test sample and a labeled compound having a binding activity to the protein are contacted;

(a) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 2, 4, 6, 28, 40 or 59;

(b) a DNA comprising the nucleotide sequence of SEQ ID NO: 1, 3, 5, 27, 39, 41, 54 or 58;

(c) a DNA hybridizing to a DNA comprising the nucleotide sequence of SEQ ID NO: 1, 3, 5, 27, 39, 41, 54 or 58 under stringent conditions;

(d) a DNA encoding a protein comprising an amino acid sequence of SEQ ID NO: 2, 4, 6, 28, 40 or 59, wherein one or more amino acids have been added, deleted, substituted and/or inserted; and

(e) a DNA amplified using primers (SEQ ID NO: 29 and 31 or SEQ ID NO: 29 and 30).

(2) a process wherein a labeled compound which binds to the protein is detected; and

(3) a process wherein a test sample which reduces the labeled compound which binds to the protein is selected.

/3

The term "stringent conditions" means, for example, hybridization in 4xSSC at a temperature of 65 degrees Celsius followed by washing with 0.1xSSC at a temperature of 65 degrees Celsius for one hour. Alternatively, stringent conditions refer to hybridization in 4xSSC with 50% formamide at a temperature of 42 degrees Celsius. Other acceptable conditions may be hybridization in PerfectHyb™ solution (TOYOBO) at a temperature of 65 degrees Celsius for 2.5 hours, followed by washing with (1) 2xSSC, 0.05%SDS at a temperature of 25 degrees Celsius for five minutes; (2) 2xSSC, 0.05%SDS at a temperature of 25 degrees Celsius for 15 minutes; and (3) 0.1xSSC, 0.1% SDS at a temperature of 50 degrees Celsius for 20 minutes.

The term "protein comprising an amino acid sequence in which one or more amino acids have been added, deleted, substituted and/or inserted" can be prepared by methods known to those skilled in the art, such as site-directed mutagenesis (Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Further, such mutations may also occur naturally. The number of amino acids to be mutated is not particularly restricted so long as the binding activity with the labeled compound can be retained. The number of amino acids to be mutated is typically 30 or less, preferably ten or less, and more preferably three or less. The position of the mutated amino acids also is not particularly restricted so long as the above-described activity can be retained.

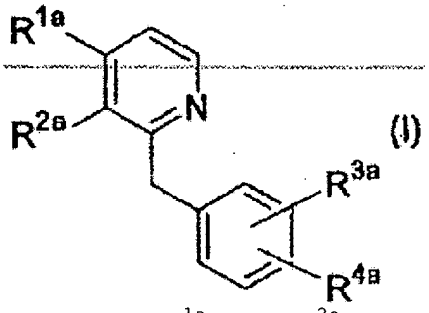
The proteins and protein mutants prepared using the above-described hybridization techniques normally have high homology (for example, 60% or higher, 70% or higher, 80% or higher, 90% or higher, or 95% or higher homology) to proteins consisting of the amino acid sequence of SEQ ID NO: 2, 4, 6, 28, 40 or 59 and the amino acid sequence itself. The amino acid sequence homology can be determined

using a BLASTx program (at the amino acid level; Altschul et al., J: Mol. Biol. 215:403-410, 1990). The program is based on the BLAST algorithm of Karlin and Altschul (Proc. Natl. Acad. Sci. USA 87:2264-2268, 1990; Proc. Natl. Acad. Sci. USA 90: 5873-5877, 1993). When the amino acid sequences are analyzed using BLASTX, parameters of, for example, score = 50 and wordlength = 3 are used.

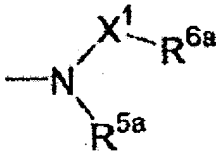
/4

Alternatively, when the Gapped BLAST program is used, the amino acid sequences can be analyzed by the method described by Altschul et al. (Nucleic Acids Res. 25: 3389-3402, 1997). When the BLAST and Gapped BLAST programs are used, the default parameter values for each program are used. Specific procedures for these analyses are known in the art (http://www.ncbi.nlm.nih.gov).

2. The method in accordance with item 1, wherein the labeled compound having a binding activity to the protein is represented by the following formula (I):



[wherein R^{1a} and R^{2a} may be the same or different and each represents a hydrogen atom, a halogen atom, a hydroxyl group, a nitro group, a cyano group, a trifluoromethyl group, a trifluoromethoxy group, a C_{1-6} alkyl group which may be substituted, a C_{2-6} alkenyl group, a C_{2-6} alkynyl group, a C_{1-6} alkoxy group or a group that can be represented by the following formula:

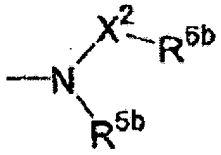


(wherein X^1 represents a single bond, a carbonyl group or a group that can be represented by formula: $-S(O)_2-$; R^{5a} and R^{6a} may be the same or different and each represents a hydrogen atom or a C_{1-6} alkyl group which may be substituted). Alternatively, R^{1a} and R^{2a} may together form a condensed ring selected from a group of a benzene ring which may be substituted, a pyridine ring which may be

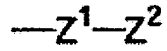
substituted, a pyrrole ring which may be substituted, a thiophene ring which may be substituted, a furan ring which may be substituted, a pyridazine ring which may be substituted, a pyrimidine ring which may be substituted, a pyrazine ring which may be substituted, an imidazole ring which may be substituted, an oxazole ring which may be substituted, a thiazole ring which may be substituted, a pyrazole ring which may be substituted, an isooxazole ring which may be substituted, an isothiazole ring which may be substituted, a cyclohexane ring which may be substituted and a cyclopentane ring which may be substituted;

/5

R^{3a} and R^{4a} may be the same or different and each represents a hydrogen atom, a halogen atom, a hydroxyl group, a nitro group, a cyano group, a carboxyl group, a formyl group, a hydroxyimino group, a trifluoromethyl group, a trifluoromethoxy group, a C_{1-6} alkyl group, a C_{1-6} alkoxy group, a C_{2-6} alkenyl group, a C_{2-6} alkynyl group, a group that can be represented by the formula: $-C(O)NR^{7a}R^{7b}$ (wherein R^{7a} and R^{7b} may be the same or different and each represents a hydrogen atom or a C_{1-6} alkyl group), the formula: $-CO_2R^{7a}$ (wherein R^{7a} is the same as above), the formula: $-S(O)_nR^{7a}$ (wherein n donates an integer of 0 to 2. R^{7a} is the same as above), the formula: $-S(O)_2NR^{7a}R^{7b}$ (wherein R^{7a} and R^{7b} are the same as above) and the following formula:



(wherein X^2 represents a single bond, a carbonyl group or a group that can be represented by formula: $-S(O)_2-$; R^{5a} and R^{6a} may be the same or different and each represents a hydrogen atom, a C_{1-6} alkyl group which may be substituted or a C_{6-14} aryl group which may be substituted), or a group that can be represented by the following formula:



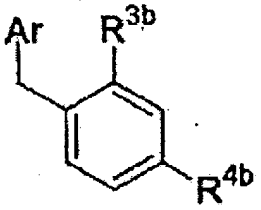
(wherein Z^1 represents a single bond, an oxygen atom, a vinylene group or an ethynylene group; Z^2 represents a single bond or a C_{1-6} alkyl group which may be substituted by 0 to 4 substituents). R^{3a} and R^{4a} may together represent a methylenedioxy group or a 1,2-ethylenedioxy group, or may together form a condensed ring selected from a group of a benzene ring which may be substituted, a pyridine ring which may be substituted, a pyrrole ring which may be substituted, a thiophene ring which may be substituted, a

furan ring which may be substituted, a pyridazine ring which may be substituted, a pyrimidine ring which may be

/6

substituted, a pyrazine ring which may be substituted, an imidazole ring which may be substituted, an oxazole ring which may be substituted, a thiazole ring which may be substituted, a pyrazole ring which may be substituted, an isooxazole ring which may be substituted, an isothiazole ring which may be substituted, a cyclohexane ring which may be substituted and a cyclopentane ring which may be substituted unless R^{1a} and R^{2a} identically represent a hydrogen atom].

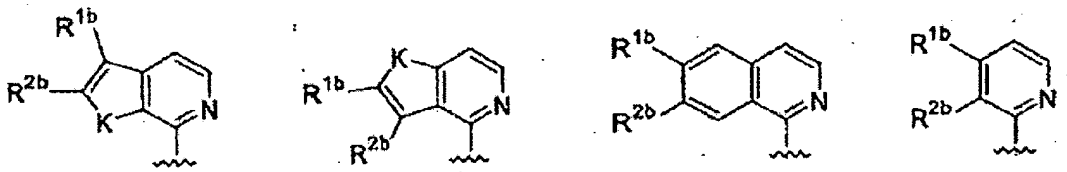
3. The method in accordance with item 1, wherein the labeled compound having a binding activity to the protein is represented by the following formula (II):



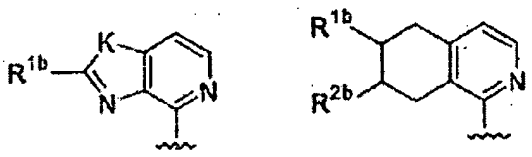
(II)

[wherein Ar represents a substituent selected from a group of (IIIa) to (IIIf):

(IIIa) (IIIb) (IIIc) (IIId)



(IIIe) (IIIf)

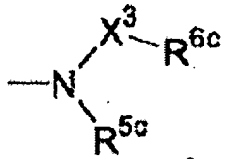


(wherein K represents a sulfur atom, an oxygen atom or a group that can be represented by the formula: -NH-;

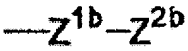
/7

R^{1b} and R^{2b} may be the same or different and each represents a hydrogen atom, a halogen atom, a hydroxyl group, a nitro group, a cyano group, a trifluoromethyl group, a trifluoromethoxy group, a C_{1-6} alkyl group which may be

substituted, a C_{1-6} alkoxy group which may be substituted, a group that can be represented by the following formula:



(wherein X^3 represents a single bond, a carbonyl group or a group that can be represented by formula: $-S(O)_2-$; R^{5c} and R^{6c} may be the same or different and each represents a hydrogen atom or a C_{1-6} alkyl group which may be substituted), or a group that can be represented by formula: $-X^4-R^{8a}$ (wherein X^4 represents a single bond, an oxygen atom or a sulfur atom; R^{8a} represents a C_{1-6} alkyl group, a C_{2-6} alkenyl group, a C_{2-6} alkynyl group, a C_{3-8} cycloalkyl group or a C_{3-8} cycloalkenyl group. Further, R^{1b} and R^{2b} may together form a methylenedioxy group or a 1,2-ethylenedioxy group);
R^{3b} and R^{4b} may be the same or different and each represents a hydrogen atom, a halogen atom, a hydroxyl group, a nitro group, a cyano group, a carboxyl group, a formyl group, a hydroxyimino group, a trifluoromethyl group, a trifluoromethoxy group, a C_{1-6} alkyl group, a C_{1-6} alkoxy group, a C_{2-6} alkenyl group, a C_{2-6} alkynyl group or a group that can be represented by the following formula:

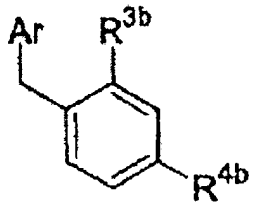


(wherein Z^{1b} represents a vinylene group or an ethynylene group;
Z^{2b} represents a single bond or a C_{1-6} alkyl group which may be substituted by 0 to 4 substituents);
unless (1) Ar can be represented by the above formula (IIId) in which both R^{1b} and R^{2b} represent a hydrogen atom, (2) at least either R^{3b} or R^{4b} represents a hydrogen atom and the other represents a hydrogen atom, a methoxy group, a hydroxyl group, a methyl group, a benzyloxy group or a halogen atom, and Ar can be represented by the above formula (IIIc) in which both R^{1b} and R^{2b} represent a hydrogen atom or a methoxy group, (3) at least either R^{3b} or R^{4b} represents a hydrogen atom and the other represents a hydrogen atom, a hydroxyl group, a methoxy group or a benzyloxy group, and Ar can be represented by the formula

/8

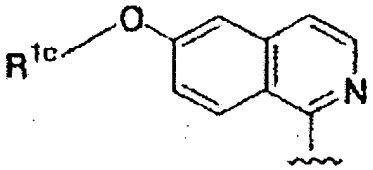
(IIIc) in which both R^{1b} and R^{2b} represent a hydroxyl group or a benzyloxy group, or (4) Ar can be represented by the above formula (IIId) in which R^{1b} represents a hydrogen atom and R^{2b} represents a formyl group, hydroxymethyl group or methoxycarbonyl group].

4. The method in accordance with item 1, wherein the labeled compound having a binding activity to the protein is represented by the following formula (II):



(II)

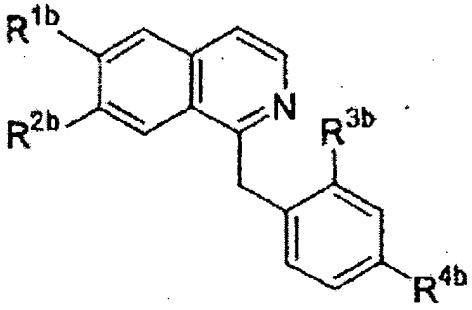
[wherein Ar can be represented by the following formula:



(IIIg)

(wherein R^{1c} represents a hydrogen atom, a C_{1-6} alkyl group which may be substituted or a benzyl group) unless R^{3b} represents a hydrogen atom].

5. The method in accordance with item 1, wherein the labeled compound having a binding activity to the protein is represented by the following formula (IIIc2):

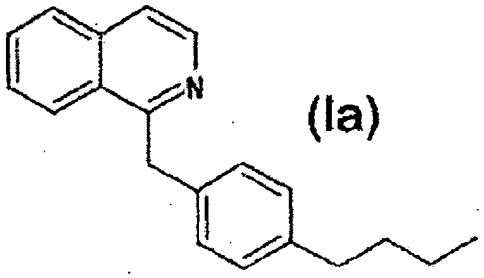


(IIIc2)

/9

[wherein R^{1b} and R^{2b} are the same as above unless (1) R^{1b} can be represented by the formula: R^{1c}-O- (wherein R^{1c} is the same as above), R^{2b} represents a hydrogen atom and R^{3b} represents a hydrogen atom, (2) at least either R^{3b} or R^{4b} represents a hydrogen atom and the other represents a hydrogen atom, a methoxy group, a hydroxyl group, a methyl group, a benzyloxy group or a halogen atom, and both R^{1b} and R^{2b} represent a hydrogen atom or a methoxy atom, (3) at least either R^{3b} or R^{4b} represents a hydrogen atom and the other represents a hydrogen atom, a hydroxyl group, a methoxy group or a benzyloxy group, and both R^{1b} and R^{2b} represent a hydroxyl group or a benzyloxy group].

6. The method in accordance with item 1, wherein the labeled compound having a binding activity to the protein is represented by the following formula (Ia):



(Ia)

7. the method in accordance with any of the items 1 through 6, comprising a process (4) which determines whether the selected test sample inhibits the process of transporting a GPI-anchored protein to a fungal cell wall or whether the test sample inhibits the expression of a GPI-anchored protein on a fungal cell surface.

The terms and symbols used throughout the present specification will now be defined, with a more detailed description of the present invention.

Several of the structural formulae given for compounds throughout the present specification will represent a specific isomer for convenience, but the invention is not limited to such specific isomers and encompasses all isomers and isomer mixtures, including geometric isomers, asymmetric carbon-derived optical isomers, stereoisomers and tautomers, implied by the structures of the compounds, of which any isomer or mixture thereof may be used.

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The compounds of the present invention therefore may include those having asymmetric carbons in their molecules and existing as optically active forms or racemic forms, and all such compounds are encompassed by the invention without restrictions. The compounds of the present invention may be crystalline or noncrystalline; there are also no restrictions on any crystalline polymorphism of the compounds, and any crystal forms may be used alone or in a mixture; and the compounds of the present invention also encompasses anhydrates and hydrates.

The term "C_{1-6} alkyl group" as used herein refers to linear or branched alkyl groups having 1 to 6 carbon atoms, with known specific examples including a methyl group, an ethyl group, an n-propyl group, an isopropyl group, an n-

butyl group, an isobutyl group, a tert-butyl group, an n-pentyl group, an isopentyl group, a neopentyl group, an n-hexyl group, a 1-methylpropyl group, a 1,2-dimethylpropyl group, a 2-ethylpropyl group, a 1-methyl-2-ethylpropyl group, a 1-ethyl-2-methylpropyl group, a 1,1,2-trimethylpropyl group, a 1-methylbutyl group, a 2-methylbutyl group, a 1,1-dimethylbutyl group, a 2,2-dimethylbutyl group, a 2-ethylbutyl group, a 1,3-dimethylbutyl group, a 2-methylpentyl group and a 3-methylpentyl group.

The term "C_{2-6} alkenyl group" as used herein refers to linear or branched alkenyl groups having 2 to 6 carbon atoms, with known specific examples including a vinyl group, an aryl group, a 1-propenyl group, an isopropenyl group, a 1-buten-1-yl group, a 1-buten-2-yl group, a 1-buten-3-yl group, a 2-buten-1-yl group and a 2-buten-2-yl group. The term "C_{2-6} alkynyl group" as used herein refers to linear or branched alkynyl groups having 2 to 6 carbon atoms, with known specific examples including an ethynyl group, a 1-propynyl group, a 2-propynyl group, a butynyl group, a pentynyl group and a hexynyl group.

The term "C_{1-6} alkoxy group" as used herein refers to oxy groups having the above-described "C_{1-6} alkyl group", /11 with known specific examples including a methoxy group, an ethoxy group, a propoxy group, n-propoxy group, an isopropoxy group, an n-butoxy group, an isobutoxy group, a sec-butoxy group, a t-butoxy group, an n-pentyloxy group, an i-pentyloxy group, a sec-pentyloxy group, a t-pentyloxy group, an neopentyloxy group, a 1-methylbutoxy group, a 2-mehtylbutoxy group, a 1,1-dimethylpropoxy group, a 1,2-dimethylpropoxy group, an n-hexyloxy group, an i-hexyloxy group, a 1-methylpentyloxy group, a 2-methylpentyloxy group, a 3-methylpentyloxy group, a 1,1-dimethylbutoxy group, a 1,2-dimethylbutoxy group, a 2,2-dimethylbutoxy group, a 1,3-dimethylbutoxy group, a 2,3-dimethylbutoxy group, a 3,3-dimethylbutoxy group, a 1-ethylbutoxy group, a 2-ethylbutoxy group, a 1,1,2-trimethylpropoxy group, a 1,2,2-trimethylpropoxy group, a 1-ethyl-1-methylpropoxy group and a 1-ethyl-2-methylpropoxy group.

The term "C_{6-14} aryl group" as used herein refers to aromatic ring groups having 6 to 14 carbon atoms, with known specific examples including a phenyl group, a 1-

naphthyl group, a 2-naphthyl group, an as-indacenyl group, an s-indacenyl group and an acenaphtylenyl group.

The term "halogen atom" as used herein refers to a fluorine atom, a chlorine atom, a bromine atom and an iodine atom.

The term "may be substituted" as used herein refers to "may be substituted by one or a plurality of substituents in an appropriate combination at a substitutable site", with known specific examples including a hydrogen atom, a halogen atom, a nitro group, a cyano group, a hydroxyl group, a mercapto group, a hydroxylalkyl group, a carboxyl group, a C_{1-6} alkoxycarbonyl group, a C_{2-7} acylamino group, a C_{1-6} alkylamino group, a pyridyl group, a C_{1-6} alkylsulfinyl group, a C_{1-6} alkylsulfonyl group, a C_{1-6} alkylsulfamoyl group, a C_{1-6} alkylsulfinamoyl, a C_{1-6} alkylsulfenamoyl group, a tetrahydropyranyl group, a C_{1-6} alkylcarbamoyl group and a group that can be represented by the formula: $-X^4-R^{8a}$ (wherein X^4 represents a single bond, an oxygen atom or a sulfur atom;

/12

R^{8a} represents a C_{1-6} alkyl group, a C_{2-6} alkenyl group, a C_{2-6} alkynyl group, a C_{6-14} aryl group, a C_{3-8} cycloalkyl group or a C_{3-8} cycloalkenyl group).

The term "may be substituted by 0 to 4 substituents" as used herein refers to "may be substituted by 1 or 4 substituents in an appropriate combination at a substitutable site", with known examples of the substituents being the same as above.

The methods for preparing the GWT1 gene products ("GWT1 protein", hereafter) (1), the binding assay for labeled compounds (2) and synthesizing the compounds that can be represented by the formula (I) (3) are exampled in the following:

1. Methods for preparing the GWT1 protein

The GWT1 protein is prepared from a fungal membrane fraction, preferably that of S. cerevisiae, C. albicans, S. pombe, A. fumigatus or C . neoformans, and more preferably S . cerevisiae. The binding assay may be performed by using the prepared membrane fraction directly or after purification. The binding assay can be readily carried out by introducing the DNA of the nucleotide sequence of SEQ ID

NO: 1, 3, 5, 27, 39, 41, 54 or 58 into fungal cells to overexpress the GWT1 protein, but the present invention is not restricted by it. The procedure using S. cerevisiae is specifically described in the following:

(1) Introduction of the GWT1 gene

The GWT1 gene can be prepared by carrying out PCR using fungal DNAs as templates, and primers with a design based on a nucleotide sequence of SEQ ID NO: 1, 3, 5, 27, 39, 41, 54 or 58.

The GWT1 expression plasmid is prepared by inserting an appropriate promoter and terminator, such as a GAPDH promoter and a GAPDH terminator derived from pKT10 (Tanaka et al., Mol. Cell Biol., 10: 4303-4313, 1990), into the multi-cloning site of an expression vector that functions in S. cerevisiae, such as YEp352, and inserting the GWT1 gene into the expression vector. S. cerevisiae cells of, for example, G2-10 strain, are cultured while shaking in an appropriate medium, such as yeast extract-polypeptone-dextrose (YPD) medium, at an appropriate temperature, for example, at a temperature of 30 degrees Celsius, and the fungal cells are harvested at the late logarithmic growth phase. After washing, GWT1 expression plasmids are introduced into S. cerevisiae cells, for example, by the lithium acetate method. The lithium acetate method is described in the Users Manual attached to YEAST MAKER ™ Yeast Transformation System (manufactured by Clontech). A GWT1-overexpressing strain and an empty vector-introduced strain can be obtained by culturing the cells in SD(ura-) medium at a temperature of 30 degrees Celsius for two days.

/13

Expression vectors and gene introduction methods for fungi other than S. cerevisiae are described in: Igarashi et al., Nature 353: 80-83, 1991, for S. pombe expression vector pcL, and methods for introducing the vectors; Pla J et al., Yeast, 12: 1677-1702, 1996 , for C. albicans expression vector pRM 10, and methods for introducing these vectors; Punt PJ et al., GENE, 56: 117-124, 1987, for A. fumigatus expression vector pAN7-1, and methods for introducing these vectors; and Monden P et al., FEMS Microbiol. Lett., 187: 41-45, 2000, for C. neoformans expression vector pPM8, and methods for introducing these vectors.

(2) Method for preparing the membrane fraction

S. cerevisiae cells into which the GWT1 gene has been introduced are cultured while being shaken in an appropriate medium, such as SD(ura-) liquid medium, at an appropriate temperature, such as a temperature of 30 degrees Celsius, and the fungal cells are harvested during the middle logarithmic growth phase. After being washed, the fungal cells are suspended in an adequate amount (for example, three times the amount of the fungal cells) of homogenization buffer (for example, 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, Complete™; manufactured by Roche), and an adequate amount (for example, four times the amount of the fungal cells) of glass beads is added to the suspension. The samples are vortexed and placed on ice; and these procedures are repeated in order to disrupt the fungal cells.

1 mL of homogenization buffer is added to the sample and the samples are then centrifuged, for example, at a rotational speed of 2,500 rpm, for five minutes in order to precipitate glass beads and fungal cells which have not been disrupted. The resulting supernatant is taken to another tube and then centrifuged at a rotational speed of 135,000 rpm for 10 minutes in order to precipitate the membrane fraction containing organelles (total membrane fraction). The precipitate is further suspended in 1 mL of a binding buffer (for example, 0.1 M Phosphate buffer, pH 7.0, 0.05% Tween 20, Complete™ (manufactured by Roche)) and then centrifuged at a rotational speed of 2,500 rpm for one minute in order to remove the components which are not suspended, and the supernatant is then centrifuged at a rotational speed of 15,000 rpm for five minutes. The precipitate is further suspended in 150 to 650 µL of binding buffer in order to obtain a membrane fraction.

/14

Membrane fractions from fungal cells other than S. cerevisiae can be prepared by the methods as described in: Yoko-o et al., Eur. J. Biochem. 257: 630-637 (1998) for S. pombe; Sentandreu M et al., J. Bacteriol., 180: 282-289 (1998) for C. albicans; Mouyna I et al., J. Biol. Chem., 275: 14882-14889 (2000) for A. fumigatus; and Thompson JR et al., J. Bacteriol., 181: 444-453 (1999) for C. neoformans.

Alternatively, GWT1 protein can be prepared by expressing it in cells other than fungal cells, such as mammalian cells, insect cells and E. coli cells.

When mammalian cells are used, a membrane fraction can be prepared by inserting GWT1 ligated to an overexpression vector having a CMV promoter into the mammalian cells and then carrying out the method described in Petaja-Repo et al., J. Biol. Chem., 276: 4416-23 (2001).

When insect cells are used, a membrane fraction can be prepared by preparing GWT1-expressing insect cells (such as Sf9 cells) using a baculovirus expression kit, for example, BAC-TO-BAC Baculovirus Expression system (manufactured by Invitrogen); and then using the cells in order to carry out the method described in Okamoto et al., J. Biol. Chem., 276: 742-751 (2001).

When E. coli is used, GWT1 protein is prepared by inserting GWT1 into an E. coli expression vector, for example, pGEX (manufactured by Pharmacia); and then introducing the vector into E. coli cells, such as BL21.

2. Binding assay methods

(1) Synthesis of labeled compound

The labeled compound is prepared from a compound that has been confirmed to bind to GWT1 proteins. Any compounds which can bind to GWT1 proteins can be used, with known preferred examples including the labeled compounds according to the compounds that can be represented by the following formula (I):

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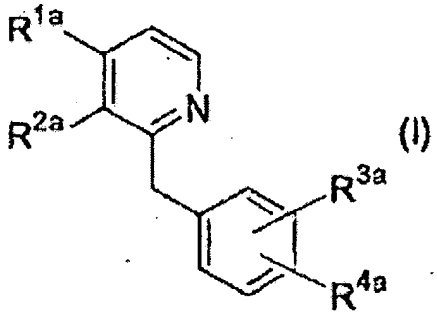
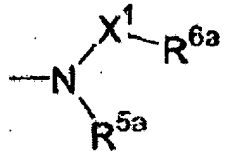


(I)

[wherein R^{1a} and R^{2a} identically or independently represent a hydrogen atom, halogen atom, a hydroxyl group, a nitro group, a cyano group, a trifluoromethyl group, a trifluoromethoxy group, a C_{1-6} alkyl group which may be

substituted, a C_{2-6} alkenyl group, a C_{2-6} alkynyl group, a C_{1-6} alkoxy group or a group that can be represented by the following formula:

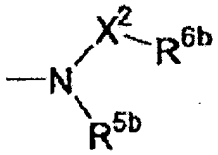


(wherein X^1 represents a single bond, a carbonyl group or a group that can be represented by $-S(O)_2-$;
R^{5a} and R^{6a} may be the same or different and each represents a hydrogen atom or a C_{1-6} alkyl group which may be substituted). Alternatively, R^{1a} and R^{2a} may together form a condensed ring selected from a group including a benzene ring which may be substituted, a pyridine ring which may be substituted, a pyrrole ring which may be substituted, a thiophene ring which may be substituted, a furan ring which may be substituted, a pyridazine ring which may be substituted, a pyrimidine ring which may be substituted, a pyrazine ring which may be substituted, an imidazole ring which may be substituted, an oxazole ring which may be substituted, a thiazole ring which may be substituted, a pyrazole ring which may be substituted, an isooxazole ring which may be substituted, an isothiazole ring which may be substituted, a cyclohexane ring which may be substituted and a cyclopentane ring which may be substituted;

R^{3a} and R^{4a} may be the same or different and each represents a hydrogen atom, a halogen atom, a hydroxyl

/16

group, a nitro group, a cyano group, a carboxyl group, a formyl group, a hydroxyimino group, a trifluoromethyl group, a trifluoromethoxy group, a C_{1-6} alkyl group, a C_{1-6} alkoxy group, a C_{2-6} alkenyl group, a C_{2-6} alkynyl group, a group that can be represented by the formula: $-C(O)NR^{7a}R^{7b}$ (wherein R^{7a} and R^{7b} may be the same or different and each represents a hydrogen atom or a C_{1-6} alkyl group), the formula: $-CO_2R^{7a}$ (wherein R^{7a} is the same as above), the formula: $-S(O)_nR^{7a}$ (wherein n donates an integer of 0 to 2. R^{7a} is the same as above), the formula: $-S(O)_2NR^{7a}R^{7b}$ (wherein R^{7a} and R^{7b} are the same as above) and the following formula:



(wherein X^2 represents a single bond, a carbonyl group or a group that can be represented by formula: $-S(O)_2-$; R^{5a} and R^{6a} may be the same or different and each represents a hydrogen atom, a C_{1-6} alkyl group which may be substituted or a C_{6-14} aryl group which may be substituted), or a group that can be represented by the following formula:

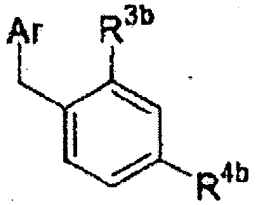
$$—Z^1—Z^2$$

(wherein Z^1 represents a single bond, an oxygen atom, a vinylene group or an ethynylene group; Z^2 represents a single bond or a C_{1-6} alkyl group which may be substituted by 0 to 4 substituents). R^{3a} and R^{4a} may together represent a methylenedioxy group or a 1,2-ethylenedioxy group, or may together form a condensed ring selected from a group of a benzene ring which may be substituted, a pyridine ring which may be substituted, a pyrrole ring which may be substituted, a thiophene ring which may be substituted, a furan ring which may be substituted, a pyridazine ring which may be substituted, a pyrimidine ring which may be substituted, a pyrazine ring which may be substituted, an imidazole ring which may be substituted, an oxazole ring which may be substituted, a thiazole ring which may be substituted, a pyrazole ring which may be substituted, an isooxazole ring which may be substituted, an isothiazole ring which may be substituted, a cyclohexane ring which may be substituted and a cyclopentane ring which may be

/17

substituted unless both R^{1a} and R^{2a} represent a hydrogen atom],

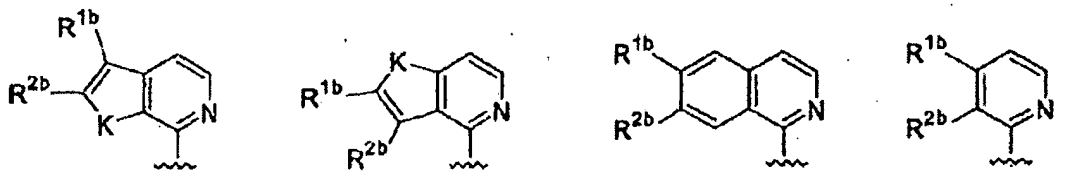
compounds that can be represented by the following general formula (II):



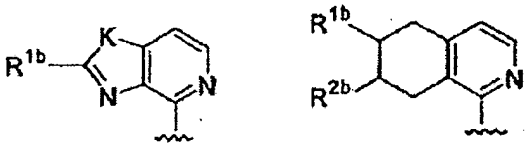
(II)

[wherein Ar represents a substituent selected from a group of (IIIa) to (IIIf):

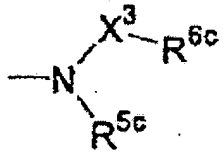
(IIIa) (IIIb) (IIIc) (IIId)



(IIIe) (IIIf)



(wherein K represents a sulfur atom, an oxygen atom or a group that can be represented by the formula: -NH-; R^{1b} and R^{2b} may be the same or different and each represents a hydrogen atom, a halogen atom, a hydroxyl group, a nitro group, a cyano group, a trifluoromethyl group, a trifluoromethoxy group, a C_{1-6} alkyl group which may be substituted, a C_{1-6} alkoxy group which may be substituted, a group that can be represented by the following formula:



(wherein X^3 represents a single bond, a carbonyl group or a group that can be represented by formula: $-S(O)_2-$; R^{5c} and

/18

R^{6c} may be the same or different and each represents a hydrogen atom or a C_{1-6} alkyl group which may be substituted), or a group that can be represented by formula: $-X^4-R^{8a}$ (wherein X^4 represents a single bond, an oxygen atom or a sulfur atom; R^{8a} represents a C_{1-6} alkyl group, a C_{2-6} alkenyl group, a C_{2-6} alkynyl group, a C_{3-8} cycloalkyl group or a C_{3-8} cycloalkenyl group. Further, R^{1b} and R^{2b} may together form a methylenedioxy group or a 1,2-ethylenedioxy group);
R^{3b} and R^{4b} may be the same or different and each represents a hydrogen atom, a halogen atom, a hydroxyl group, a nitro group, a cyano group, a carboxyl group, a formyl group, a hydroxyimino group, a trifluoromethyl group, a trifluoromethoxy group, a C_{1-6} alkyl group, a C_{1-6} alkoxy

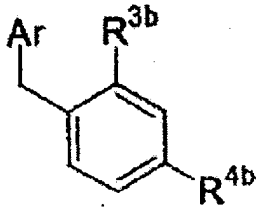
group, a C_{2-6} alkenyl group, a C_{2-6} alkynyl group or a group that can be represented by the following formula:

$-Z^{1b}-Z^{2b}$

(wherein Z^{1b} represents a vinylene group or an ethynylene group;

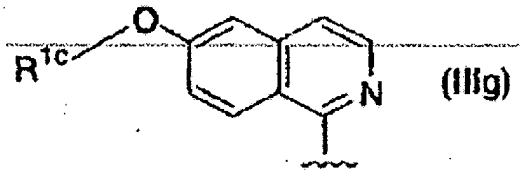
Z^{2b} represents a single bond or a C_{1-6} alkyl group which may be substituted by 0 to 4 substituents); unless (1) Ar can be represented by the above formula (IIId) in which both R^{1b} and R^{2b} represent hydrogen atoms, (2) at least either R^{3b} or R^{4b} represents a hydrogen atom and the other represents a hydrogen atom, a methoxy group, a hydroxyl group, a methyl group, a benzyloxy group or a halogen atom, and Ar can be represented by the above formula (IIIc) in which both R^{1b} and R^{2b} represent hydrogen atoms or methoxy groups, (3) at least either R^{3b} or R^{4b} represents a hydrogen atom and the other represents a hydrogen atom, a hydroxyl group, a methoxy group or a benzyloxy group, and Ar can be represented by the formula (IIIc) in which both R^{1b} and R^{2b} represent a hydroxyl group or a benzyloxy group, or (4) Ar can be represented by the above formula (IIId) in which R^{1b} represents a hydrogen atom and R^{2b} represents a formyl group, hydroxymethyl group or methoxycarbonyl group],
compounds that can be represented by the following general formula (II):

/19



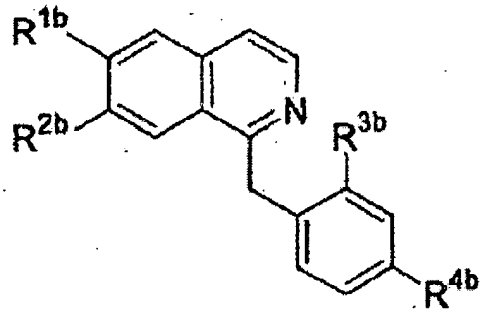
(II)

[wherein Ar can be represented by the following formula:



(IIIg)

(wherein R^{1c} represents a hydrogen atom, a C_{1-6} alkyl group which may be substituted or a benzyl group) unless R^{3b} represents a hydrogen atom],
compounds that can be represented by the following general formula (IIIc2):



(IIIc2)

[wherein R^{1b} and R^{2b} are the same as above. unless (1) R^{1b} can be represented by the formula: R^{1c}-O- (wherein R^{1c} is the same as above), R^{2b} represents a hydrogen atom and R^{3b} represents a hydrogen atom, (2) at least either R^{3b} or R^{4b} represents a hydrogen atom and the other represents a hydrogen atom, a methoxy group, a hydroxyl group, a methyl group, a benzyloxy group or a halogen atom, and both R^{1b} and R^{2b} represent a hydrogen atom or a methoxy atom, (3) at least either R^{3b} or R^{4b} represents a hydrogen atom and the other represents a hydrogen atom, a hydroxyl group, a methoxy group or a benzyloxy group, and both R^{1b} and R^{2b} represent a hydroxyl group or a benzyloxy group], and

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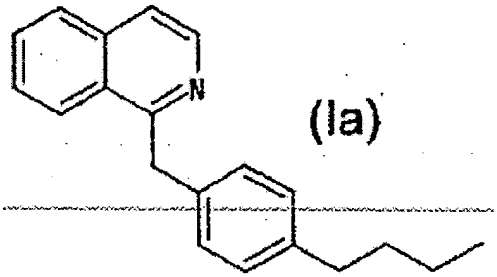
compounds that can be represented by the following general formula (Ia):



(Ia)

Of the above-described compounds, the compounds having a binding activity to the GWT1 protein are preferred and the compounds that show an activity at a reporter system in S. cerevisiae are more preferred as a labeled compound. Further, the compounds having a binding activity to the GWT1 protein that have been found by the inventive method can be labeled and used as a labeled compound.

Although any labeling methods can be used, the compound is preferably labeled with a radioisotope, and more preferably with ^{3}H. The radio-labeled compound can be prepared by typical production methods using a radioactive compound as a starting material. Alternatively, ^{3}H labeling can be achieved using an ^{3}H exchange reaction.

(2) Confirmation of specific binding

The labeled compound is added to the prepared membrane fraction and the mixture is then allowed to stand on ice for an appropriate time, for example, one to two hours, in order to carry out the binding reaction between the labeled compound and the membrane fraction. The membrane fraction is precipitated by centrifuging the mixture, for example, at a rotational speed of 15,000 rpm, for three minutes. The operation in which the precipitate is resuspended in a binding buffer and the suspension is centrifuged is repeated appropriately (twice) in order to remove any unbound labeled compound. The precipitate is again suspended in the binding buffer and the resulting suspension is transferred into a scintillation vial, and a scintillator is added in order to measure the radioactivity using a liquid scintillation counter.

The specific binding of the labeled compound to the GWT1 protein can be confirmed by assessing whether binding of the labeled compound is inhibited by adding an excessive amount (ten times or more) of unlabeled compound, and

/21

whether the compound binds negligibly to membrane fractions prepared from fungal cells which do not express the GWT1 protein.

(3) Binding inhibition of a labeled compound by a test sample

A test sample and the labeled compound are added to the prepared membrane fraction, and the mixture is allowed to stand on ice for an appropriate period of time, for example, for one to two hours, in order to carry out the reaction with the membrane fraction. The membrane fraction is precipitated by centrifuging the mixture, for example, at a rotational speed of 15,000 rpm, for three minutes. The operation in which the precipitate is resuspended in the binding buffer and the suspension is centrifuged is then repeated appropriately (twice) in order to remove any unbound labeled compound. The precipitate is again suspended in the binding buffer and the resulting suspension is transferred into a scintillation vial, and a scintillator is added in order to measure the radioactivity using a liquid scintillation counter.

When the binding of the labeled compound to the membrane fraction is inhibited in the presence of the test sample, the test sample is confirmed to have the activity of binding to the GWT1 protein.

The test sample which has been confirmed to have a binding activity is preferably assessed to verify whether the test sample inhibits the process of transporting a GPI-anchored protein to a fungal cell wall or whether the test sample inhibits the expression of a GPI-anchored protein on a fungal cell surface. When the test sample is found to inhibit the process of transporting a GPI-anchored protein to a fungal cell wall or inhibit the expression of a GPI-anchored protein on a fungal cell surface, the test sample can be a strong candidate for an antifungal agent.

Whether a test sample inhibits the process of transporting a GPI-anchored protein to a fungal cell wall or whether a test sample inhibits the expression of a GPI-anchored protein on a fungal cell surface can be confirmed by: (1) by using a reporter enzyme; (2) by using an antibody that reacts with the surface glycoprotein of fungal cell walls; (3) by using the protein's ability to adhere to animal cells; or (4) by observing fungal cells under a light microscope or an electron microscope.

Whether a test sample inhibits the process of transporting a GPI-anchored protein to a fungal cell wall or whether a test sample inhibits the expression of a GPI-anchored protein on a fungal cell surface can be confirmed by the following methods (1) to (4), preferably in a combination thereof, and, when the degree of inhibition is reduced or prevented by overexpressing a protein encoded by the DNA described in the present specification, the test

/22

sample can be confirmed to affect the process of transporting a GPI-anchored protein to a fungal cell wall.

The methods (1) to (4) are explained in the following:

(1) Method using a reporter enzyme

The process that transports GPI-anchored proteins to the cell wall can be quantified using a tracer experiment wherein a GPI-anchored protein is labeled with a radioactive isotope in order to obtain the fungal cell wall fraction and then immunoprecipitated using an antibody

against the GPI-anchored protein. Alternatively, quantification can be more readily performed as follows: the C-terminal sequence, which is considered to act as a transport signal and is commonly observed among GPI-anchored proteins, can be expressed as a fusion protein with an easily measurable enzyme (reporter enzyme) in order to obtain the fungal cell wall fraction, and a reporter system that measures the enzyme activity of each fraction can be quantified (Van Berkel MAA et al., FEBS Letters, 349: 135-138 (1994)). A method using a reporter enzyme is explained in the following, but does not restrict the present invention in any way.

Firstly, a reporter gene is constructed and then introduced into fungi. The reporter gene is constructed by linking a promoter sequence that acts on fungi with DNAs that respectively encode a signal sequence, a reporter enzyme and a GPI-anchored protein C-terminal sequence in such a way that the reading frames can be matched. Known examples of the promoter sequence include GAL10 and ENO1. Known examples of the signal sequence include α-factor, invertase and lysozyme. Known examples of reporter enzymes include β-lactamase, lysozyme, alkaline phosphatase and β-galactosidase. Green Fluorescence Protein (GFP), which has no enzyme activity but can be easily detected, can also be used. Known examples of GPI-anchored protein C-terminal sequences include the α-agglutinin C-terminal sequence and the CWP2 C-terminal sequence. Furthermore, it is preferable to insert an appropriate selection marker, such as LEU2 and URA3, into the vector comprising the constructed reporter gene.

The constructed reporter gene is inserted into fungi using an appropriate method, such as the lithium acetate /23 method (Gietz D etal., Nucl. Acids Res. 20: 1425 (1992)), and the fungi are then cultured, as appropriate, using a method that suits the selection marker (e. g. using Leu^- medium for LEU2 and Ura^- medium for URA3), and the fungi into which the DNA has been introduced are then selected.

Whether a test sample affects the process of transporting a GPI-anchored protein to a fungal cell wall can be confirmed by the following method:

The reporter gene-introduced fungi are cultured under appropriate conditions, for example, at a temperature of 30

degrees Celsius for 48 hours, in the presence of a test sample. After culturing, the culture supernatant is centrifuged in order to measure the reporter enzyme activity of the culture supernatant fraction. The remaining cell fraction is washed, and the cell wall components are then separated using an appropriate method, such as degrading the cell wall glucan with glucanase, in order to measure the reporter enzyme activity of the cell wall fraction and cytoplasmic fraction. Further, in order to simplify the assay, after centrifugation, the amount of reporter enzyme derived from the culture supernatant fraction remaining in the cell fraction can be calculated, without washing the cells, using a proportional calculation, and the amount of reporter enzyme in the cell fraction can be obtained by subtracting the calculated amount from the amount of reporter enzyme of the cell fraction.

If the test sample shows the activity of increasing reporter enzyme activity within the culture supernatant fraction (activity per cell) or the activity of decreasing the reporter enzyme activity in the cell wall fraction (activity per cell), the test sample is confirmed to have affected the transport process of GPI-anchored proteins to the cell wall.

(2) Method using an antibody that reacts with the surface glycoprotein of fungal cell walls

Whether a test sample affects the expression of a GPI-anchored protein on a fungal cell surface can be confirmed by quantifying the GPI-anchored protein in the fungal cell wall using an antibody that reacts with the GPI-anchored protein.

The antibodies can be obtained by predicting the antigenic determinant using the amino acid sequence of, for example, a GPI-anchored protein, such as α-agglutinin, Cwp2p and Alslp (Chen MH et al., J. Biol. Chem. , 270: 26168-26177 (1995); Van Der Vaat JM et al., J. Bacteriol., 177: 3104-3110 (1995); Hoyer LL et al., Mol. Microbiol., 15: 39-54 (1995)),

/24

then synthesizing the peptide of the region, binding it to an antigenic substance, such as heterologous proteins, and then immunizing a rabbit in order to obtain a polyclonal antibody, or immunizing a mouse in order to obtain a

monoclonal antibody. Further, a rabbit polyclonal antibody which is against the Alslp peptide is preferred.

Alternatively, a monoclonal antibody which is against a GPI-anchored protein may be obtained by immunizing mice with fungi, preferably fungi in which a GPI-anchored protein such as α-agglutinin, Cwp2p and Alslp, is overexpressed (in some cases by immunizing further with a partially purified GPI-anchored protein), and then selecting the produced antibody of the resulting clones using ELISA or Western blot analysis.

Whether a test sample affects the process of transporting a GPI-anchored protein to a cell wall and reduces the amount of the GPI-anchor-derived proteins in the cell wall can be confirmed by the following method:

Fungi are cultured under appropriate conditions, for example, at a temperature of 30 degrees Celsius for 48 hours, in the presence of a test sample. The cultured fungi are collected by centrifugation and the cells are disrupted, preferably using glass beads. The washed, disrupted cells are preferably subjected to centrifugal extraction with SDS, and then the precipitate is washed. After extraction, the disrupted cells are treated with an enzyme that degrades glucan, preferably glucanase, and the centrifuged supernatant thereof is taken as the GPI-anchored protein sample.

The anti-Alslp peptide antibody is coated onto a 96-well plate by overnight incubation at a temperature of 4 degrees Celsius. The plate is washed with a washing solution, preferably a PBS comprising 0.05% Tween 20 (PBST), and then blocked using a reagent that blocks the non-specific adsorption sites of the 96-well plate, preferably a protein, such as BSA and gelatine, more preferably BlockAce. The plate is again washed with a washing solution, preferably PBST, and an appropriately diluted GPI-anchored protein sample is then added, and the reaction is allowed to be reacted for an appropriate time, such as for two hours, at room temperature. After washing with a washing solution, preferably with PBST, an antibody against the enzyme-labeled C. albicans, preferably HRP-labeled anti-Candida antibody, is reacted for an appropriate time, such as for two hours, at room temperature. The labeling method may be enzyme labeling or radioactive isotope labeling. After washing with a washing solution, preferably PBST, the

amount of Alslp in the GPI-anchored protein sample is calculated by a method according to the type of label, i.e., /25 for an enzyme label, by adding a substrate solution and then, upon stopping the reaction, measuring absorbance at 490 nm.

(3) Method using the ability to adhere to animal cells

Whether a test sample affects the expression of a GPI-anchored protein on a fungal cell surface can be confirmed by measuring the activity of the GPI-anchored protein in the fungal cell wall, and preferably by measuring the ability of fungi to adhere to animal cells. Besides the activity of Alslp and Hwplp in adhesion to animal cells, known examples of the GPI-anchored protein activity include a-agglutinin in mating and Flolp in yeast aggregation. A method using the ability of fungi to adhere to animal cells is explained in the following, but does not restrict the present invention in any way.

A fungus with the ability to adhere to cells is used, and C. albicans is preferably used. For mammalian cells, cells that adhere to the fungus, preferably intestinal epithelial cells, are used. The mammalian cells are cultured and fixed using an appropriate method, such as ethanol fixation. The test sample and the fungi which have been incubated for an appropriate time, such as at a temperature of 30 degrees Celsius for 48 hours, are inoculated and cultured for a set time, for example, at a temperature of 30 degrees Celsius for one hour, and the culture supernatant is then removed, and the cells are washed with a buffer and overlaid with agar media, such as Sabouraud Dextrose Agar Medium (Difco). After culturing at a temperature of 30 degrees Celsius overnight, the number of colonies is counted and the adhesion rate is calculated.

As compared to fungi which have not been treated with the compound, if a test sample is observed to have the activity of decreasing the number of colonies formed by cell adhesion, the test sample is confirmed to have affected the transport process of GPI-anchored proteins to the cell wall.

(4) Method for observing fungi using an electron microscope or an optical microscope

Whether a test sample affects the expression of a GPI-anchored protein on a fungal cell surface can be confirmed by observing the structure of the fungal cell wall using an electron microscope.

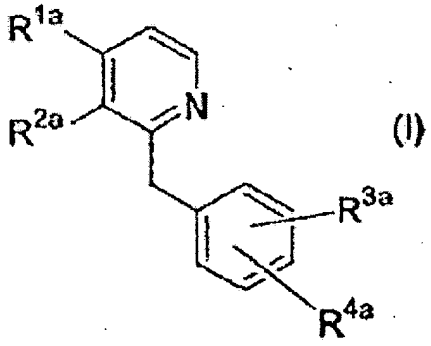
/26

In the presence of a test sample, a fungus, such as C. albicans, is cultured for a set time, such as at a temperature of 30 degrees Celsius for 48 hours, and the ultrafine morphological structure thereof is observed using a transmission electron microscope. The observation using a transmission electron microscope can be carried out, for example, by the method according to the Electron Microscope Chart Manual (published by Medical Publishing Center). The flocculent fibrous structure of the outermost layer of a fungal cell having a high electron density which can be observed by a transmission electron microscope is considered to be a surface glycoprotein layer including GPI-anchored proteins as a constituent and are therefore not affected by other existing antifungal agents. As compared to the untreated fungi, when this structure disappears leaving only a slight layer with a high electron density, the test sample is confirmed to have affected the transport process of GPI-anchored proteins to the cell wall.

When observation under both a transmission electron microscope and an optical microscope reveals greatly swollen fungal cells and the inhibited budding (division) of fungal cells, the test sample is confirmed to have affected the cell wall.

3. Method for synthesizing the compounds that can be represented by the formula (I)

The compounds of the present invention that can be represented by the formula (I):



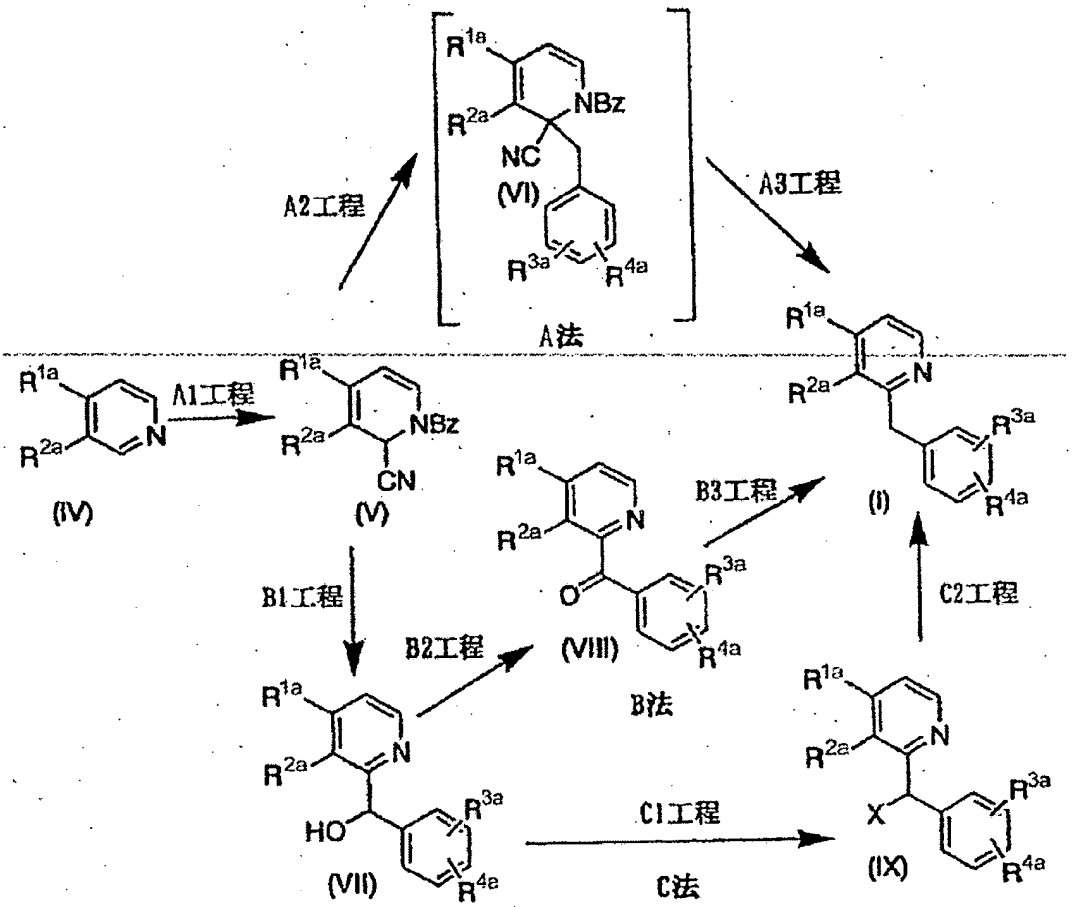
(I)

(wherein the symbols have the same meaning as defined above) can be synthesized by utilizing conventional organic

chemical reactions; for example, it can be synthesized by the following methods:

Production Method (1)

/27



(wherein X represents a leaving group, such as a halogen group and an acryl group. R^{3c} and R^{3a} have the same meaning as defined above. Other symbols in the formulae have the same meaning as defined above.)

Process A1 Reaction for producing the Reissert compound (V). The compound can be produced based on the reaction conditions in accordance with the literature, such as in Org. Synth., VI, 115(1988); Heretocycles, 36(11), 2489(1993); J. Chem. Soc. (C), 666(1969); or J. Heterocycl. Chem., 29(5), 1165(1192). Known specific examples of the reagents include a combination of benzoyl chloride and potassium cyanide.

Process A2 A process for alkylation. The compound (VI) can be produced by reacting the compound (V) with a substituted benzyl halide derivative, a substituted benzylmethane sulfonate derivative, or such in the presence of a base. Known examples of the base include sodium hydride and sodium hydroxide.

/28

Process A3 A process for hydrolysis reaction. The compound (I) can be produced by hydrolysis of the compound (VI) in the presence of a base.

Method A is a method for producing the compound (I) via Process A1, Process A2 and Process A3.

Process B1 A process for conversion of the compound (V) to the compound (VII). The compound (VII) can be produced by reacting the compound (V) with substituted benzaldehyde in the presence of a base and a phase-transfer catalyst. Known examples of the base include sodium hydroxide and potassium hydroxide. Known examples of the phase-transfer catalyst include triethylbenzylammonium chloride.

Process B2 A process for oxidation of the alcohol to the ketone. The ketone derivative (VIII) can be produced by using an oxidizing agent and a condition conventionally used for the oxidation reaction of an alcohol to a ketone. Known examples of the oxidizing agents include manganese dioxide, chromium dioxide and benzoquinone.

Process B3 A process for the reduction of the ketone to the methylene. The methylene derivative (I) can be produced by using a conventionally used combination of reducing agents for the reduction reaction of the ketone derivative (VIII) to the methylene derivative (I). Known examples of the combination of the reducing agents include hydrazine hydrate and sodium hydroxide or potassium hydroxide, and triethylsilane and boron trifluoride, or trifluoromethanesulfonic acid.

Method B is a method for producing the compound (I) via Process A1, Process B1, Process B2, and Process B3.

Process C1 A process for halogenations or acylation of the hydroxyl group. The compound (IX) can be produced by reacting a halogenating agent or an acylating agent with the compound (VII). Known examples of halogenating agent include thionyl chloride, concentrated hydrochloric acid,

and phosphorus tribromide. Further, known examples of the acylating agent include acid halides, such as acetyl chloride, and acid anhydrides, such as acetic anhydride.

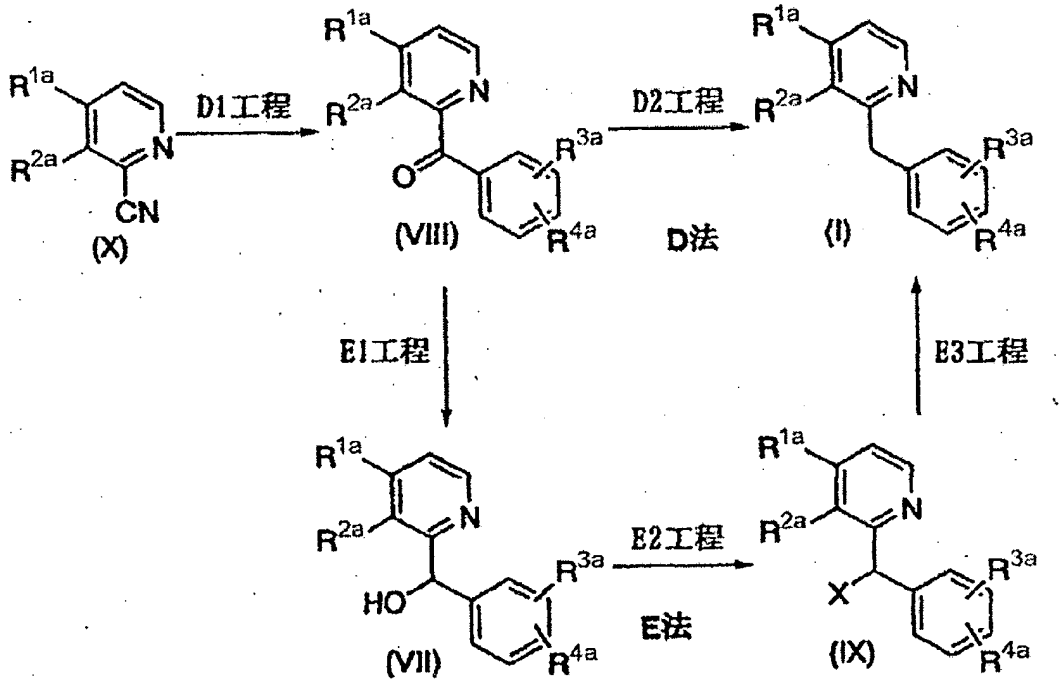
/29

Process C2 A process for a reductive elimination reaction of the halogen group or the acyl group. The compound (I) can be produced by hydroelimination of the compound (IX), for example, by using a catalyst. Known examples of the catalyst include palladium-carbon.

Method C is a method for producing the compound (I) via Process A1, Process B1, Process C1, and Process C2.

Production Method (2)

The compound of the present invention that can be represented by the formula (I) can also be synthesized by the following method:



(wherein X represents a leaving group, such as a halogen group and an acryl group. Other symbols in the formulae have the same meaning as defined above.)

Process D1 A process for a Grignard reaction and a subsequent acid hydrolysis reaction. The compound (VIII) can be produced by reacting the compound (X) with a

substituted or unsubstituted Grignard reagent, followed by hydrolysis in the presence of an acid.

Process D2 The methylene derivative (I) can be produced from the ketone derivative (VIII) by conditions similar to those of Process B3.

/30

Method D is a method for producing the compound (I) via Process D1 and Process D2.

Process E1 A process for a reduction reaction from the ketone to the alcohol. The compound (VII) can be produced from the compound (VIII) using a reducing agent and conditions conventionally used for the reduction reaction of a ketone to an alcohol. Known examples of the reducing agent include sodium borohydride and lithium aluminum hydride.

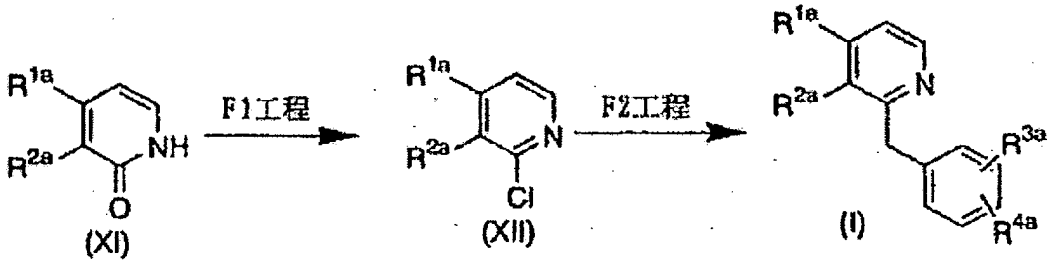
Process E2 Under conditions similar to those of Process C1, the halogenated or acylated derivative (IX) can be produced from the alcohol derivative (VII).

Process E3 Under conditions for the reductive elimination reaction similar to those of Process C2, the compound (I) can be produced from the compound (IX).

Method E is a method for producing the compound (I) via Process D1, Process E1, Process E2, and Process E3.

Production Method (3)

The compound of the present invention that can be represented by the formula (I) can also be synthesized by the following method:



(wherein the symbols in the formulae have the same meaning as defined above.)

Process F1 A process for a chlorination reaction. The compound (XII) can be produced by reacting the compound (XI) with a chlorinating agent. Known examples of the chlorinating agent include phosphorus oxychloride and thionyl chloride.

Process F2 A process for the coupling reaction with a Grignard reagent. The compound (I) can be produced by

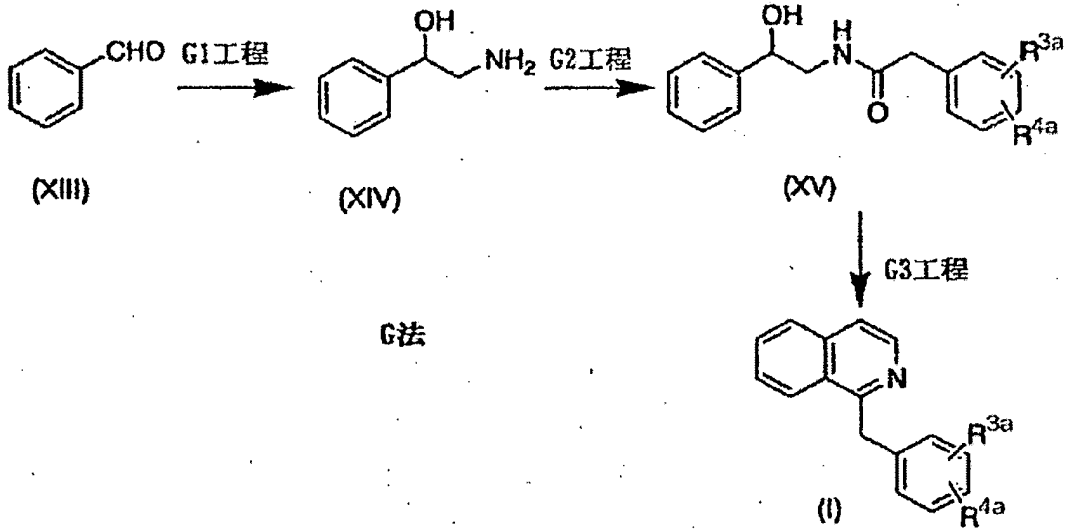
/31

reacting the compound (XII) with a substituted or unsubstituted benzyl Grignard reagent in the presence of a catalyst, based on the reaction conditions in accordance with the literature, such as Arch. Pharm, 314, 156(1981). Known examples of the catalyst include [1,1'-bis(diphenylphosphino)ferrocene]dichloro nickel(II).

Method F is a method for producing the compound (I) via Process F1 and Process F2.

Production Method (4)

The compound of the present invention of the formula (I), where in R^{1a} and R^{2a} together form a condensed ring, such as a benzene ring, a pyridine ring, a pyrrole ring, a thiophene ring, a furan ring, a cyclohexane ring, or a cyclopentane ring, can be synthesized by the following method:



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(wherein the symbols in the formulae have the same meaning as defined above.)

The production method in which the isoquinoline ring is formed is shown below as an example.

Process G1 A process for the condensation reaction and the subsequent reduction reaction. The compound (XIV) can be produced by a condensation reaction between the substituted or unsubstituted benzaldehyde derivative (XIII) and nitromethane, followed by the reduction of the nitro group. Known examples of the reagent that can be used for the reduction of the nitro group include a combination of palladium-carbon and ammonium formate, and lithium aluminum hydride.

Process G2 An amide bond formation reaction.

/32

The compound (XV) can be produced by reacting the compound (XIV) and a substituted or unsubstituted phenylacetyl chloride with a coupling reagent for an amide bond formation reaction. Known examples of the coupling reagent include a combination of N,N'-dicyclohezylcarbodiimide and N-hydroxysuccinimide, a combination of N,N'-dicyclohezylcarbodiimide and N-hydroxybenzotriazole, and 1,1'-carbonyldiimidazole.

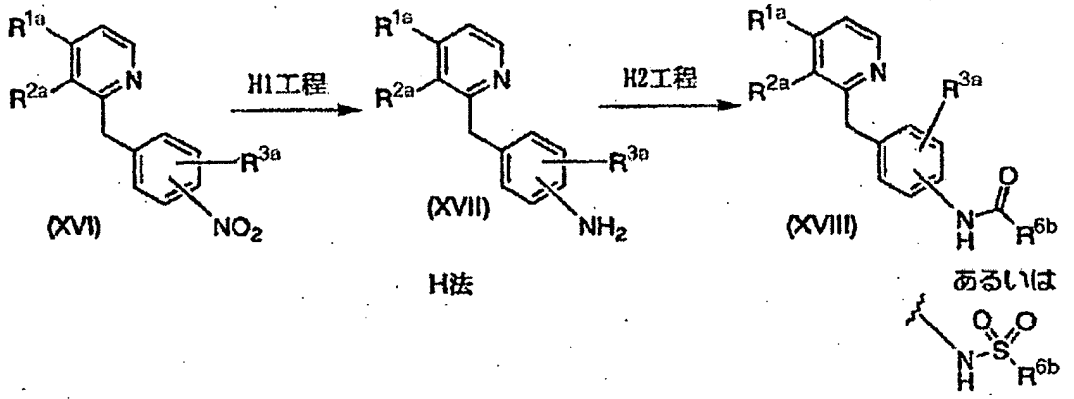
Process G3 A process for cyclization reaction. The compound (XV) can be produced based on the reaction conditions in accordance with the literature, such as Organic Reaction, 6, 74(1951); J. Hetetocyclic Chem., 30, 1581(1993). Known examples of the reagents that can be used for the reaction include phosphorus oxychloride and polyphosphoric acid.

Method G is a method for producing the compound (I) via Process G1, Process G2, and Process G3.

Production Method (5-1)

Replacement of the substituent R^{3a} or R^{4a} of the compound (I) synthesized by the above-described production method.

(5-1) Replacement of the substituent with an amino group, an amide group, a sulphonamide group, etc.



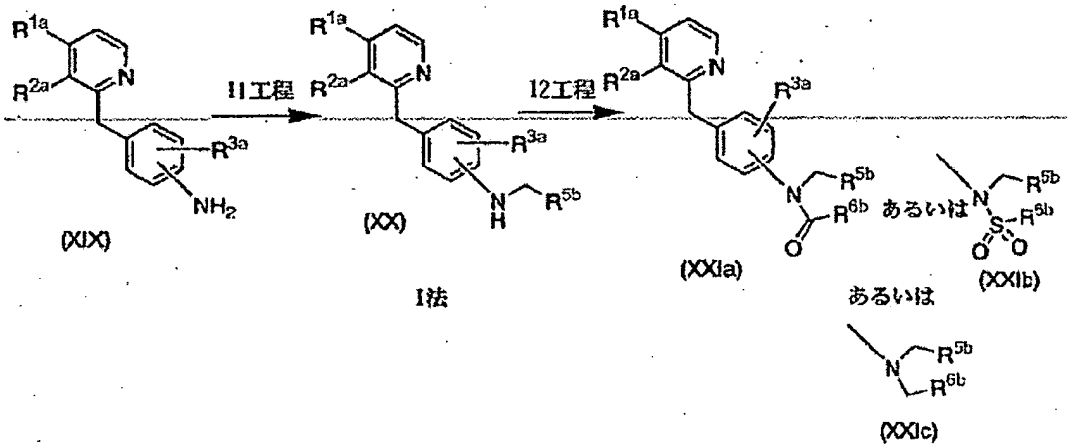
(wherein the symbols in the formulae have the same meaning as defined above.)

Process H1 A reduction reaction of the nitro group. The compound (XVII) can be produced by reducing the compound (XVI) with a conventionally used method for reduction of a nitro group. Known examples of the reduction method include catalytic hydrogenation reduction by palladium-carbon or palladium hydroxide, and reduction by iron-ammonium chloride, iron-hydrochloric acid or iron-acetic acid.

/33

Process H2 A process for acylation or sulfonylation reaction. The compound (XVIII) can be produced by treating the compound (XVII) with an acid chloride or acid anhydride.

Method H is a method for producing the compound (XVIII) via Process H1 and Process H2.



(wherein the symbols in the formulae have the same meaning as defined above.)

Process I1 A process for the reductive amination reaction. The compound (XX) can be produced from the

compound (XIX) and a substituted or unsubstituted aldehyde based on the reaction conditions in accordance with the literature, such as J. Am. Chem. Soc., 93, 2897(1971); Comprehensive Organic Synthese, 8, 25(1991); Tetrahedron, 40, 1783(1984); and Tetrahedron, 41, 5307(1985). Known examples of the reductive amination reagent include sodium triacetoxyhydroborate, sodium cyanotrihydroborate, borane-pyridine complex, and palladium-carbon/hydrogen.

Process I2 A process for acylation, sulfonylation, or reductive amination reaction. The compound (XVIa) or the compound (XXIb) can be produced from the compound (XX) using an acid chloride or an acid anhydride. The compound (XXIc) can be produced by carrying out a reductive amination reaction similar to that of Process I1.

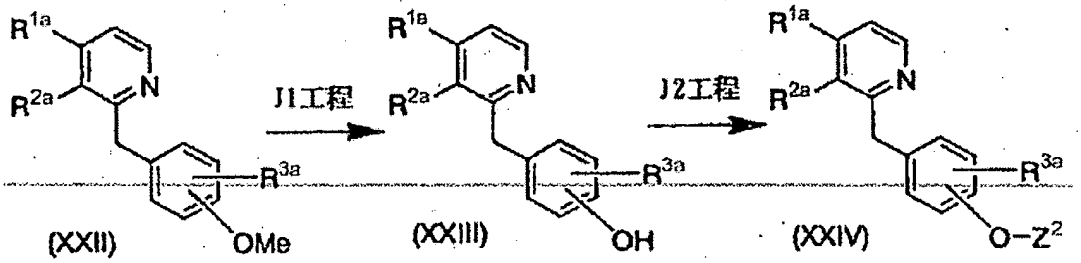
Method I is a method for producing the compound (XXIa), the compound (XXIb), or the compound (XXIc) via Process I1 and Process I2.

/34

Production Method (5-2)

Replacement of the substituent R^{3a} or R^{4a} of the compound (I) synthesized by the above-described production method.

(5-2) Replacement of the substituent with a hydroxyl group, an alkoxy group, etc.



(wherein the symbols in the formulae have the same meaning as defined above.)

Process J1 The compound (XXIII) can be produced from the compound (XXII) by a demethylation reaction based on the reaction conditions according to the literature, such as Bull. Chem. Soc. Jpn., 44, 1986(1971); Org. Synth., Collect. Vol. V, 412(1073); J. Am. Chem. Soc., 78, 1380(1956); or J. Org. Chem., 42, 2761(1977). Known examples of the reagent that can be used for the demethylation reaction include 47% aqueous hydrobromic acid

solution, boron tribromide, pyridine hydrochloride, and iodotrimethylsilane.

Process J2 A process for the alkylation reaction. The compound (XXIV) can be produced by reacting the compound (XXIII) with a substituted or unsubstituted alkyl halide, a substituted or unsubstituted alkylmethane sulfonate, etc, in the presence of a case.

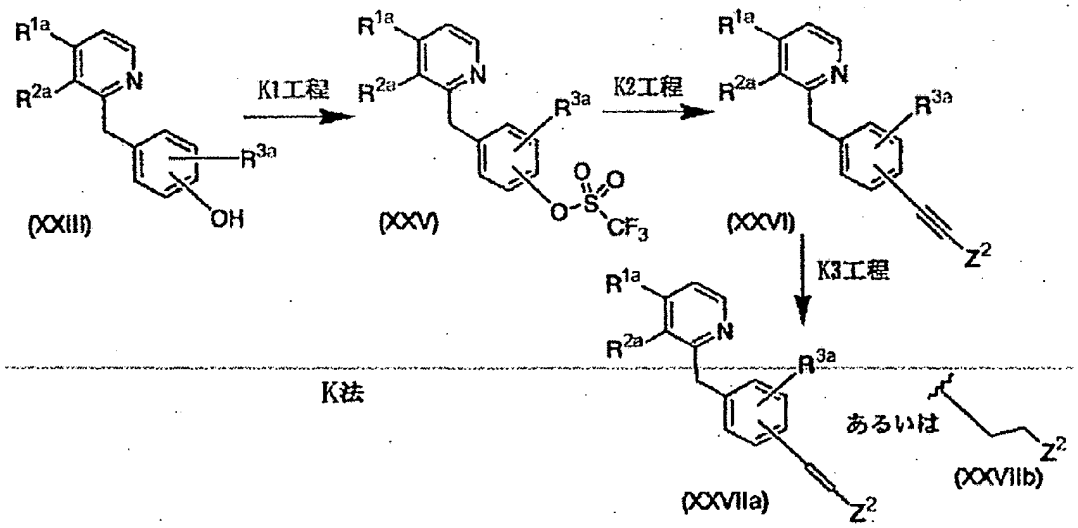
Method J is a method for producing the compound (XXIV) via Process J1 and Process J2.

Production Method (5-3)

Replacement of the substituent R^{3a} or R^{4a} of the compound (I) synthesized by the above-described production method.

/35

(5-3) Replacement of the substituent with a vinylene group, an ethylene group, an alkyl group, etc.



(wherein the symbols in the formulae have the same meaning as defined above.)

Process K1 A process for the triflation reaction. The compound (XXV) can be produced by reacting the compound (XXIII) with trifluoromethane sulfonic acid anhydride in the present of a base.

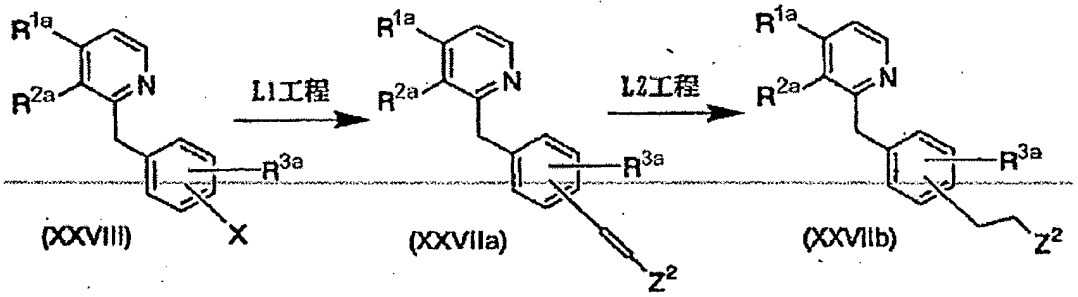
Process K2 A process for the coupling reaction with an alkyne. The compound (XXVI) can be produced by coupling the compound (XXV) with an alkyne derivative in the presence of

a palladium phosphine complex, copper iodide, and a base. Known examples of the reagents that produce the palladium phosphine complex in the reaction system include a combination of palladium-carbon and triphenylphosphine, tetrakistriphenylphosphine palladium (0) and triphenylphosphine, dichlorobistriphenylphosphine palladium (II), palladium (II) acetate and tri(o-tolyl)phosphine, and palladium (II) acetate and 1,1'-bis(diphenylphosphino)ferrocene. Known examples of the base include triethylamine, piperidine, pyridine, and potassium carbonate. Depending on the reaction, lithium chloride may be used.

Process K3 A process for the reduction reaction of the unsaturated hydrocarbon.

/36

The compound (XXVIIa) or the compound (XXVIIb) can be produced from the compound (XXVI), for example, by catalytic hydrogenation reaction using a catalyst. Known examples of the catalyst include palladium-carbon, palladium hydroxide, platinum oxide, and palladium-carbon-calcium carbonate.



(wherein the symbols in the formulae have the same meaning as defined above.)

Process L1 A process of a coupling reaction (Heck Reaction) with the alkene. The compound (XXVIIa) can be produced from the compound (XXVIII) using a catalyst (e.g., palladium complex and its ligand), based on the reaction condition in accordance with the literature, such as J. Org. Chem., 37, 2320(1972); Org. Reactions., 27, 345(1982); Comprehensive Organic Synthesis, Vol. 4, 833(1991); Palladium Reagents and Catalysts, 125(1995); Chem. Commun., 1287(1984); Tetrahedron Lett, 26, 2667(1985); and Tetrahedron Lett, 31, 2463(1990). Known examples of the combination of the catalysts that can be used for the reaction (palladium and its ligand) include palladium (II) acetate and 1,1'-bis(diphenylphosphino)ferrocene and palladium (II) acetate and tri(o-tolyl)phosphine. Known

example of the tertiary base include triethylamine, diisopropylethylamine, and 1,8-diazabicyclo[5.4.0]-7-undecene. X of the compound (XXVIII) denotes a leaving group, such as a halogen group and trifluoromethanesulfonyloxy group.

Process L2 The compound (XXVIIb) can be produced from the compound (XXVIIa) according to the conditions for a reduction reaction of an unsaturated hydrocarbon, similar to that of process K3.

/37

Method L is a method for producing the compound (XXVIIa) by Process L1, followed by producing the compound (XXVIIb) by Process L2.

Various isomers of the compounds represented by the above-described formula (I) of the present invention can be purified and isolated using ordinary separation techniques (such as recrystallization and chromatography).

Brief Description of the Drawings

FIG. 1 is a schematic diagram of the process that transports GPI-anchored proteins to the cell wall. A GPI-anchored protein is first anchored to the plasma membrane, and then transported to the cell wall.

FIG. 2 is a graph showing the activity of the above-described compound (Ia) in the S. cerevisiae reporter system. In the presence of the above-described compound (Ia) at a concentration of 0.39 to 1.56 μg/mL, cephalosporinase activity increased in the culture supernatant fraction and decreased in the cell wall fraction, and at a concentration of 3.13 μg/mL or more, growth inhibition was observed.

FIG. 3 is a graph showing the effect of the above-described compound (Ia) on the adhesion of C. albicans to animal cells. Even at a concentration of 1.56 μg/mL in which growth inhibition cannot be observed, adhesion of C. albicans to animal cells was inhibited by approximately a half.

FIG. 4 is a graph showing the effect of the above-described compound (Ia) on different amounts of Alslp

antigen of C. albicans. In the presence of the above-described compound (Ia) at a concentration of 0.1 to 0.39 μg/mL, the amount of the Alslp antigen increased in the culture supernatant fraction and the amount of the antigen decreased in the cell wall fraction.

FIG. 5 is a photograph showing the Southern Blot of C. albicans genomic DNA analysis using the GWT1 gene as a probe. A single band was observed at 6.5 kb with EcoRI, at 4.0 kb with HindIII, at 2.0 kb with EcoRI-HindIII, and at 2.5 kb with EcoRI-PstI, and the homologue of the resistant gene to the above-described compound (Ia) in C. albicans was expected to exist as a single gene.

/38

FIG. 6 is a graph showing the activity of the above-described compound (Ia) in the S. cerevisiae that overexpressed the GWT1 gene product. In S. cerevisiae CW63 strain ("W/T" in the Figure), even at the concentration of the above-described compound (Ia) (0.39 to 1.56 μg/mL) in which cephalosporinase activity in the culture supernatant fraction is increased, and activity in the cell wall fraction is decreased, such an effect was not observed in S. cerevisiae CW63/GWT1 strain, and in S. cerevisiae CW63 strain, even at the concentration of the above-described (>3.13 μg/mL) in which growth is inhibited, growth inhibition was not observed in S. cerevisiae CW63/GWT1 strain ("O/E" in the Figure).

FIG. 7 is a diagram in which the highly conserved regions in the proteins encoded by the GWT1 genes of S. cerevisiae, S. pombe, and C. albicans are aligned.

FIG. 8 is a graph showing the specific binding of a labeled compound 1 (1-(4-butylbenzyl) isoquinoline) to a membrane fraction that has expressed the GWT1 protein.

FIG. 9 is a graph showing the binding inhibition of a labeled compound 1 to a membrane fraction that has expressed S. cerevisiae GWT1 gene product by a test sample.

FIG. 10 is a graph showing the detected result of a compound 1 to Candida albicans GWT1 gene product.

Detailed Description of the Preferred Embodiments

The following specific Examples explain the present invention, but do not restrict it in any way.

Example 1: Construction of the Reporter Gene and Introduction thereof into S. cerevisiae

(1) Construction of the Reporter Gene where Lysozyme is the Reporter Enzyme

A lysozyme gene comprising a promoter sequence was amplified by PCR using pESH plasmid comprising the ENO1 promoter + secretion signal + the lysozyme gene (Ichikawa K et al, Biosci. Biotech. Biochem., 57(10), 1686-1690, 1993) as a template, and the oligonucleotides of SEQ ID NO: 8 and SEQ ID NO: 9 as primers, and this was subcloned into the SaII-EcoRI site of pCR-Script SK(+)(a).

/39

Further, a CWP2 gene was amplified by PCR using S. cerevisiae chromosomal DNA as a template, and the oligonucleotides of SEQ ID NO: 10 and SEQ ID NO: 11 as primers, and this was subclosed into the EcoRI-HindIII site of pUC19 (b). Similarly, CYC1 terminator was amplified by PCR using pYES2 (INVITROGEN) as a template, and the oligonucleotides of SEQ ID NO: 12 and SEQ ID NO: 13 as primers, and this was subclosed into the newly introduced NotI-KpnI site of pUC19 (c).

Next, the lysozyme gene excised with SaII-EcoRI (a), and the CWP2 gene excised with EcoRI-HindIII (b) were inserted into the SaII-HindIII cleavage site of pESH. Finally, pRLW63T was produced by excising a gene comprising the ENO1 promoter + secretion signal + lysozyme gene + CWP2 gene using BamHI-HindIII, inserting this into a pRS306 integration vector (Siforski RS et al, Genetics. 122(1):19-27, 1989), and then inserting the CYC1 terminator excised with HindIII-KpnI (c) into the HindIII-KpnI cleavage site.

(2) Construction of the Reporter Gene where Cephalosporinase is the Reporter Enzyme

DNA comprising a promoter sequence and secretion signal portion that was amplified by PCR using the above-mentioned pESH as a template, and the oligonucleotides of SEQ ID NO: 14 and SEQ ID NO: 15 as primers, and this was subclosed into the BamHI-NotI site newly introduced into pUC19 (d). Further, a cephalosporinase gene was amplified by PCR using Citrobacter freundii chromosomal DNA as a temple, and the oligonucleotides of SEQ ID NO: 16 and SEQ ID NO: 17 as primers, and this was subclosed into the NspV-XbaI site

newly introduced into pUC19 (e). Similarly, the CWP2 gene was amplified by PCR using S. cerevisiae chromosomal DNA as a template, and the oligonucleotides of SEQ ID NO: 18 and SEQ ID NO: 19 as primers, and this was subclosed into the XbaI-HindIII site of pUC19 (f).

After producing the full length ENO1 promoter + secretion signal portion by inserting the BamHI-SaII

/40

fragment of pESH into the BamHI-SaII cleavage site of a plasmid into which (d) has been inserted, the cephalosporinase gene excised with NspV-XbaI, and the CWP2 gene excised with XbaI-HindIII were inserted into the NspV-HindIII cleavage site. Next, pRCW63T was produced by excising with EcoRI-HindIII, inserting this fragment into the above-mentioned pRS306, and then inserting the CYC1 terminator into the HindIII-KpnI cleavage site.

(3) Introduction of the Reporter Gene into S. cerevisiae

S. cerevisiae G2-10 strain was cultured by shaking in 10 mL of YPD medium at a temperature of 30 degrees Celsius, and then the cells were collected at the late logarithmic growth phase ($2\text{-}5\times10^7$ cells/mL). After washing with sterilized water, the above-mentioned pRLW63T and pRCW63T were introduced by lithium acetate method that uses YEASTMAKER™ Yeast Transformation System (Clontech) (according to the YEASTMAKER™ Yeast Transformation System User Manual). pRLW63T and pRCW63T in which the URA3 gene was cleaved with EcoRV and ApaI, respectively, were used. After culturing in SD (Ura^-) medium at a temperature of 30 degrees Celsius for three days, the grown colonies were cultured in YPD medium.

When the localizations of lysozyme and cephalosporinase activities were confirmed, both activities were mainly localized in the cell wall, and the C-terminal sequence of CWP2 was confirmed to function as a transport signal to the cell wall.

Example 2: Screening of Pharmaceutical Agents by the S. cerevisiae Reporter System

Since sensitivity of the enzyme reaction is better with cephalosporinase compared to lysozyme, S. cerevisiae introduced with pRCW63T (S. cerevisiae CW63 strain) was used for the screening of compounds.

After stationary cultivation in YPD liquid medium at a temperature of 30 degrees Celsius for 48 hours, the yeast cell culture was diluted 100 times with YPD liquid medium ($3\text{-}5\times10^5$ cells/mL) and 75 µL/well aliquots thereof were inoculated into a V-bottomed 96-well plate containing 25 µL/well of a diluted test sample, and this was subjected to stationary cultivation at a temperature of 30 degrees Celsius for 48 hours. After centrifuging the plate, 25 µL of the supernatant was sampled and placed in a flat-bottomed 96-well plate, and this was used as the culture supernatant fraction.

The precipitated cells were suspended, and 75 µL/well aliquots of Zymolyase (manufactured by Seikagaku Corporation) solution prepared with 2.4 M sorbitol were added and were allowed to react at a temperature of 30 degrees Celsius for an hour.

/41

After centrifuging the plate, 10 µL of the supernatant was sampled and placed in a flat-bottomed 96-well plate, 15 µL of phosphate buffer was added, and this was used as the cell wall fraction.

The cephalosporinase activities in the medium and in the cell wall fraction were measured by adding 200 µM of nitrocefin solution to a pooled sample, and after a certain period of time, stopping the reaction with citric acid buffer, and then measuring the absorbance at 490 nm.

Further, fungal growth in the presence of the test sample was determined by visual observation.

FIG. 2 showed that, in the presence of the above-described compound (Ia) at a concentration of 0.39 to 1.56 µg/mL, cephalosporinase activity increases in the culture supernatant fraction, and the activity decreases in the cell wall fraction. In this manner, a compound that increases the cephalosporinase activity in the culture supernatant fraction, and in addition decreases the cephalosporinase activity in the cell wall fraction was considered to be a compound that inhibits the process that transports GPI-anchored proteins to the cell wall.

Example 3: Screening of Pharmaceutical Agents Using the Adhesion of Candida to Animal Cells

3 mL aliquots of IEC-18 cells (1×10^5 cells/mL in D-MEM medium (manufactured by Nissui Pharmaceutical) containing 10% fetal calf serum and 2 mM glutamine) were placed in each well of a 6-well multi-well plate. The plate was incubated in a carbon dioxide gas incubator at a temperature of 37 degrees Celsius for three days, the culture supernatant was removed, and ethanol immobilization was then carried out.

C. albicans cultured in Sabouraud Dextrose Liquid Medium containing various concentrations of the test sample at a temperature of 30 degrees Celsius for 48 hours was adjusted to 4×10^2 cells/mL, and 1 mL thereof was inoculated into each well of the plate in which the immobilized IEC-18 cells were cultured. After cultivation at a temperature of 30 degrees Celsius for an hour, the culture supernatant was removed, washed with PBS, and then 2 mL of Sabouraud Dextrose Agar Medium (Difco) was superposed. After culturing at a temperature of 30 degrees Celsius overnight, the number of colonies (CFU) that had grown was counted and the adhesion rate was calculated.

/42

FIG. 3 shows that even at a concentration of 1.56 μg/mL of the above-mentioned compound (1a), in which growth inhibition cannot be observed, adhesion of C. albicans to animal cells was inhibited to about a half. Compared to untreated C. albicans, a test sample that diminished CFU that adhered to cells was considered as a compound that inhibits the adhesion of C. albicans to animal cells.

Example 4: Screening of Pharmaceutical Agents Using the Amount of the GPI-Anchored Protein Quantified by ELISA

(1) Production of Anti-Alslp Peptide Antibody

A house rabbit was immunized with the synthetic peptide of SEQ ID NO: 20 which was conjugated with KLH. The resulting antisera were affinity-purified, and the IgG fraction was used as the anti-Alslp peptide antibody.

(2) Screening of Pharmaceutical Agents by ELISA Using Anti-Alslp Peptide Antibody

C. albicans was cultured in Sabouraud Dextrose Liquid Medium (5 mL) containing various concentrations of the test sample at a temperature of 30 degrees Celsius for 48 hours,

and the cells were collected by centrifugation, washed, and then suspended in 300 μL of Tris-HCl buffer. The suspended cells were transferred to a microtube containing glass beads, and were disrupted by repeating 10 cycles of stirring for one minute and cooling on ice for one minute. The disrupted cells that had been washed were extracted with 2% SDS at a temperature of 95 degrees Celsius for 10 minutes, centrifuged, and then the precipitate was washed 5 times with phosphate buffer. To the resulting precipitate, 0.5 mL of 5 μg/mL Zymolyase solution was added, reacted at a temperature of 37 degrees Celsius for an hour, and the centrifuged supernatant was used as the GPI-anchored protein sample.

A 96-well plate was coated with 50 μL of anti-Alslp peptide antibody (40 μg/mL) at a temperature of 4 degrees Celsius overnight. After washing 5 times with PBS containing 0.05% Tween 20 (PBST), blocking was carried out with 25% BlockAce at room temperature for two hours. After washing 3 times with PBST, 50 μL of the 2-fold serially diluted GPI-anchored protein sample was reacted at room temperature for two hours. After washing 5 times with PBST, 100 μL of 1000-fold diluted HRP-labeled anti-Candida antibody (ViroStat) was reacted at room temperature for two hours, then after washing 5 times with PBST, 75 μL of substrate solution was added. After the reaction was stopped, absorbance at 490 nm was measured.

/43

FIG. 4 shows that in the presence of the aforementioned compound (1a) at a concentration of 0.1 to 0.39 μg/mL, the amount of Alslp antigen increases in the culture supernatant fraction, and the amount of antigen decreases in the cell wall fraction. In this manner, a compound that increased the amount of Alslp in the culture supernatant, or decreased the amount of Alslp in the cell wall fraction, as quantified by ELISA, compared to the amount of Alslp in C. albicans untreated with the compound, was considered to be a compound that inhibits the process that transports GPI-anchored proteins to the cell wall in C. albicans.

Example 5: Observation of the Cell Wall of C. albicans Cultured in the Presence of a Test Sample by an Electron Microscope

C. albicans which was cultured in Sabouraud Dextrose Liquid Medium (5 mL) containing various concentrations of

the test agent at a temperature of 30 degrees Celsius for 48 hours, then centrifuged, and collected, was immobilized by potassium permanganate immobilization method, and the transmission electron microscope image thereof was observed.

The flocculent fibrous structure with high electron density was observed in the outermost layer of the cell, and was considered to be the surface layer glycoprotein layer having the GPI-anchored protein as its constituent. This flocculent fibrous structure was not influenced by other existing antifungal agents.

In C. albicans cultured in the presence of the above-mentioned compound (1a), the flocculent fibrous structure of the outermost layer of the cell having high electron density disappeared leaving a small amount of the layer with high electron density, compared to that in untreated cells. In this manner, when the flocculent fibrous structure of the outermost layer of the fungal cell having high electron density disappeared, the test sample was considered to be the compound influencing the process that transports GPI-anchored proteins to the cell wall.

Example 6: Screening of the Resistant Gene to the Above-Mentioned Compound (1a) of S. cerevisiae

The plasmid library of the S. cerevisiae gene was obtained from ATCC (Information for ATCC Number: 37323).

S. cerevisiae G2-10 strain was cultured by shaking in 10 mL of YPD medium at a temperature of 30 degrees Celsius, and then the cells were collected at the late logarithmic growth phase ($1\text{-}2\times10^7$ cells/mL). After washing the cells with sterilized water, the plasmid library of the S. cerevisiae gene was introduced by the lithium acetate method that uses YEASTMAKER™ Yeast Transformation System (Clontech) (according to YEASTMAKER™ Yeast Transformation System User Manual), and this was spread onto a SD (Leu^-) plate, and approximately 80,000 colonies were obtained.

/44

The colonies were collected and diluted, and were spread onto a SD (Leu^-) plate containing the above-mentioned compound (1a) at a concentration of 1.56 μg/ml and 3.125 μg/ml so that there were 570,000 colonies per plate. Subsequently, the resistant clone was obtained by incubation at a temperature of 37 degrees Celsius for 72 hours.

When 27 clones were picked up and plasmids were collected by the method according to METHODS IN ENZYMOLOGY, Vol. 194: 169-182 (1991), and the inserts were analyzed, all 27 clones contained the same fragment.

As a result of determining the nucleotide sequence using the ABI377 system (PE Applied Biosystems), the DNA of SEQ ID NO: 1 was found to be the DNA that confers resistance to the above-mentioned compound (1a), and was named GWT1.

Example 7: Southern Blot Analysis of a C. albicans Homologue of the S. cerevisiae GWT1 Gene

A sample was prepared by treating 25 µg of the C. albicans genomic DNA with EcoRI (TaKaRa), HindIII (TaKaRa), BamHI (TOYOBO), or PstI (New England Biolabs) (including a combination of two types of enzymes) for 16 hours, then concentrating by ethanol precipitation, and dissolving in 25 µL of sterilized water. 25 µg of genomic DNA digested with restriction enzymes was separated by 0.75% agarose gel electrophoresis method, and was transferred to a nylon membrane (GeneScreen PLUS/NEN).

A probe was produced by labeling 20 ng of the approximately 1.5 kb DNA fragment of SEQ ID NO: 1 with alpha33P-dCTP by the random primer method, and was purified using a GeneQuant column (Amersham-Pharmacia).

/45

Hybridization was carried out by soaking the membrane in 10 mL of PerfectHyb™ (TOYOBO) solution, pre-incubating at a temperature of 65 degrees Celsius for an hour, then adding the labeled probe mentioned above, and incubating at a temperature of 65 degrees Celsius for 2.5 hours. Washing was carried out with 1). 2×SSC, 0.05% SDS solution at a temperature of 25 degrees Celsius for five minutes, 2). 2×SSC, 0.05% SDS solution at a temperature of 25 degrees Celsius for 15 minutes, and 3). 0.1×SSC, 0.1% SDS solution at a temperature of 50 degrees Celsius for 20 minutes. The washed membrane was wrapped with Saran Wrap, and contacted with an Imaging Plate (FUJI) for 12 hours at room temperature, the image that had been transferred to the Imaging Plate was captured using BAS2000 (FUJI), and the resulting image was analyzed.

As a result, single bands were observed at 6.5 kb with EcoRI, 4.0 kb with HindIII, 2.0 kb with EcoRI-HindIII, and 2.5 kb with EcoRI-PstI (FIG. 5), and the homologue of the resistant gene to the above-mentioned compound (1a) of C. albicans was expected to exist as a single gene.

Example 8: Screening of the Resistant Gene to the Above-Mentioned Compound (1a) of C. albicans

The genomic library of C. albicans was produced by the method according to Navaro-Garcia F et al, Mol. Cell. Biol., 15: 2197-2206, 1995. Specifically, the genomic DNA of C. albicans was partially digested with Sau3AI, then DNA fragments around 3 to 5 kb were collected, and these were inserted into the BamHI site of YEp352 shuttle vector.

S. cerevisiae G2-10 strain was cultured by shaking in 10 mL of YPD medium at a temperature of 30 degrees Celsius, and then the cells were collected at the late logarithmic growth phase ($2\text{-}5\times10^7$ cells/mL). After washing the cells with sterilized water, a genomic library of the C. albicans was introduced by the lithium acetate method that uses YEASTMAKER™ Yeast Transformation System (Clontech) (according to YEASTMAKER™ Yeast Transformation System User Manual), and this was spread onto a SD(Ura^-) plate, and approximately 25,000 colonies were obtained. The colonies were collected and diluted, and were spread onto a SD plate containing the above-mentioned compound (1a) at a concentration of 1.56 μg/mL so that there were 500,000 colonies per plate. Subsequently, the resistant clones were obtained by incubation at a temperature of 30 degrees Celsius for six hours, and then transferred to a temperature of 37 degrees Celsius and incubated for 66 hours.

/46

When 30 clones were picked up and plasmids were collected by the method according to METHODS IN ENZYMOLOGY, Vol. 194: 169-182 (1991), and the inserts were analyzed, 28 out of 30 clones contained the same fragment.

As a result of determining the nucleotide sequence using the ABI377 system (manufactured by PE Applied Biosystems), the DNA of SEQ ID NO: 3 was found to be the DNA that confers resistance to the above-mentioned compound (1a).

Example 9: Cloning of a Homologue of the Resistant Gene to the Above-Mentioned Compound (Ia) from the Clinical Isolate of C. albicans

A PCR amplification with primers of SEQ ID NO: 21 and SEQ ID NO: 22 using, as a template, a genomic DNA that had been purified from a clinical isolate of C. albicans that had been stored by the inventors was carried out. Approximately 1.6 kb of DNA fragments were amplified from all three of the independent PCR samples, the resulting amplified fragments were purified and then subcloned into a pT7-Blue vector (Novagen) in order to determine the nucleotide sequence; thus, the DNA sequence of SEQ ID NO: 5 was discovered. The sequence was different at three positions as compared to the DNA of Example 7 (SEQ ID NO: 3)

Further, in the nucleotide sequence of the C. albicans gene determined at Stanford University Sequence Center (http://sequence-www.stanford.edu/), a homologue of the DNA of Example 7 was discovered (SEQ ID NO: 7), and the sequence was different at four positions as compared to the DNA of Example 7 (SEQ ID NO: 3)

Example 10: Construction of S. cerevisiae Overexpressing the GWT1 Gene Product

A PCR amplification with primers of SEQ ID NO: 23 and SEQ ID NO: 24 using a plasmid that had been purified from the resistant clone to the above-mentioned compound (Ia) obtained in Example 6 as a template was carried out. A PCR product cleaved with PvuII was inserted into the SalI-HindIII cleavage site of pRLW63T produced in Example 1.

/47

The entire insert was excised with BamHI-KpnI and then inserted into the MCS (multi-cloning site) of pRS304 (Sikorski RS et al, Genetics. 122(1): 19-27, 1989) in order to produce a vector for integration.

S. cerevisiae CW63 strain having a cephalosporinase gene as a reporter gene was cultured by the method according to Example 1, and TRP1 of the integration vector was cleaved with EcoRV and then the transformation was carried out by the method of Example 1. GWT1-overexpressed strain (S. cerevisiae CW63/GWT1 strain) was obtained by culturing the resulting S. cerevisiae CW63 strain in SD(Trp^-

) medium at a temperature of 30 degrees Celsius for three days.

Other than showing resistance to the above-mentioned compound (1a), GWT1-overexpressed strain was not different from the wild type strain, and was sensitive towards other antifungal agents, such as cycloheximide, benomyl, and amphotericin B.

Example 11: Construction of S. cerevisiae Mutant Lacking the GWT1 Gene

His5 cassette containing the GWT1 sequence on both ends was amplified by PCR using the his5 gene of S. pombe (Longtine MS et al, Yeast, 14: 953-961, 1998) as a template and SEQ ID NO: 25 and SEQ ID NO: 26 as primers.

S. cerevisiae G2-10 was cultured and the cells were collected by the method according to Example 1, and the above-mentioned PCR product was transformed by the method according to Example 1. A GWT1-deficient strain was thus obtained by cultivation in SD(His^-) medium at a temperature of 30 degrees Celsius for five to seven days.

Although the GWT1-deficient strain showed very slow growth, it was suggested that the growth was not affected by the above-mentioned compound (1a) and the GWT1 gene product was the target of the compound. Further, the GWT1-deficient strain indicated the following characteristics: it could not grow at high temperatures; and the cells were swollen, and, in the observation by a transmission electron microscope, the flocculent fibrous structure of the outermost layer of the fungal cell having high electron density had disappeared.

Example 12: Activity of the Above-Mentioned Compound (1a) in S. cerevisiae Overexpressing the GWT1 Gene Product

/48

The activity of the above-mentioned compound (Ia) was examined in accordance with the method described in Example 2 by using S. cerevisiae CW63/GWT1 into which S. cerevisiae strain and GWT1 gene were introduced.

As a result, no influence was observed in the S. cerevisiae CW63/GWT1 strain even at a concentration (0.39 to 1.56 μg/mL) of the above-described compound (1a) at

which cephalosporinase activity in the culture supernatant fraction was increased and the activity in the cell wall fraction was decreased in S. cerevisiae CW63 strain, and growth inhibition was not observed in the S. cerevisiae CW63/GWT1 strain even at a concentration (>3.13 μg/mL) of the above-mentioned compound (1a) at which growth was inhibited in S. cerevisiae CW63 strain (see, FIG. 6).

Example 13: Synthesis of (4-butylphenyl)(1-isoquinolyl)ketone

Under a nitrogen atmosphere, 2.29 mL of 1-bromo-4-butyl-benzene (13.0 mmol) was added to a mixture solution of 338 mg of magnesium (13.9 mmol) and 6.5 mL tetrahydrofuran with a catalytic amount of 1,2-dibromoethane as an initiator, and the resulting mixture solution was stirred under reflux for 10 minute The mixture solution was then cooled to a temperature of 0 degree Celsius and, after 1.0 g of a tetrahydrofuran solution of 1-isoquinolinecarbonitrile (6.49 mmol) was added thereto, the mixture solution was stirred for another hour at room temperature and at a temperature of 70 degrees Celsius for three hours. Subsequently, the mixture solution was cooled again to a temperature of 0 degree Celsius, and, after 2.56 mL of concentrated hydrochloric acid and 11 mL of methanol were added, the mixture solution was then refluxed for two hours. The concentrated residue was dissolved in 5 N sodium hydroxide and toluene and then filtered through celite. The toluene layer of the filtrate was divided, washed with water, dried over magnesium sulfate, and then concentrated. The residue was purified by silica gel column chromatography in order to obtain 1.72 g of the title compound.

^{1}H-NMR($CDCl_3$)δ(ppm):0.93(3H, t), 1.32-1.43(2H, m), 1.58-1.66(2H, m), 2.68(2H, t), 7.28(2H, d), 7.61(1H, td), 7.74(1H, td), 7.80(1H, d), 7.87(2H, d), 7.92(1H, d), 8.20(1H, d), 8.60(1H, d)

/49

Example 14: Synthesis of the Above-Described Compound of the Formula (1a) [1-(4-butylbenzyl)isoquinoline]

1.72 g (5.95 mmol) of the compound of Example 13, 836 mg (16.7 mmol) of hydrazine monohydrate and 769 mg (13.7 mmol) of potassium hydroxide were added to 8.5 mL of diethylene glycol, and the mixture was stirred at a

temperature of 80 degrees Celsius for an hour, at a temperature of 160 degrees Celsius for three and a half hours, and at a temperature of 200 degrees Celsius for an hour. Upon cooling to room temperature, ice water was added to the mixture and then extracted with ethyl acetate. The resulting extract was washed with water, then dried over magnesium sulfate, and concentrated. The residue was purified by silica gel column chromatography in order to obtain 914 mg of the above-described compound of the formula (1a).

^{1}H-NMR($CDCl_3$)δ(ppm):0.89(3H, t), 1.26-1.36(2H, m), 1.50-1.59(2H, m), 2.53(2H, t), 4.64(2H, s), 7.06(2H, d), 7.19(2H, d), 7.53(1H, td), 7.56(1H, d), 7.64(1H, td), 7.81(1H, d), 8.18(1H, dd,), 8.50(1H, d)

Example 15: Another Method for Producing the Above-Mentioned Compound of the Formula (1a) [1 -(4-butylbenzyl)isoquinoline]

3.6 mL of a dimethylformamide solution of 100 mg (0.38 mmol) of 1-cyano-2-benzoyl-1,2-dihydroiso-quinoline and 70 mg (0.38 mmol) of 4-n-butylben-zylchloride that had been synthesized according to the literature of Org. Synth., VI, 115(1988), was added dropwise to a dimethylformamide (1.8 mL) solution of 16 mg (0.40 mmol) of 60% sodium hydride under a nitrogen atmosphere at a temperature of -16 degrees Celsius, and the mixture was further stirred at room temperature for 30 minutes. Water was added in order to concentrate the mixture, and toluene and water were added to the residue. The toluene layer was washed with water, dried over potassium carbonate, and then concentrated. 0.63 mL of 50% aqueous sodium hydroxide solution was added to 1.6 mL of an ethanol solution of the residue, and the mixture solution was then refluxed with heat for two hours. After concentration, toluene and water were added. The toluene layer was washed with water, dried over calcium carbonate, and then concentrated. The residue was purified by silica gel column chromatography in order to obtain 18 mg of the above-described compound of the formula (1a).

/50

Example 16: Cloning of the C. albicans Homologue of the S. cerevisiae GWT1 Gene

25 µg of the C. albicans genomic DNA that had been treated with HindIII (TaKaRa) for 16 hours was separated by

0.75% agarose gel electrophoresis method, and the DNA fragments ranging in size from approximately 3.5 to 4.5 kb were collected from the gel. The collected DNA fragments were inserted into the HindIII site of the pKF3 vector (TaKaRa) in order to produce a Candida genomic library.

Using the produced library, approximately 10,000 colonies were displayed on an LB/Ampicillin plate, colony lifting was performed using a Colony/Plaque Screen (NEN) membrane, and then subjected to hybridization. A probe was produced by labeling 20 ng of the approximately 1.5 kb DNA fragment of SEQ ID NO: 1 with alpha33P-dCTP by the random primer method, and was purified using a GeneQuant column (Amersham-Pharmacia).

Hybridization was carried out by soaking the membrane in PerfectHyb™ (TOYOBO) solution, pre-incubating at a temperature of 65 degrees Celsius for an hour, then adding the labeled probe mentioned above, and incubating at a temperature of 65 degrees Celsius for 2.5 hours. Washing was carried out with 1). 2×SSC, 0.05% SDS solution at a temperature of 25 degrees Celsius for five minutes, 2). 2×SSC, 0.05% SDS solution at a temperature of 25 degrees Celsius for 15 minutes, and 3). 0.1×SSC, 0.1% SDS solution at a temperature of 50 degrees Celsius for 20 minutes. The washed membrane was wrapped with Saran Wrap, contacted with an X-RAY FILM (KONICA) for 24 hours at room temperature, and then developed. The E. coli colonies corresponding to the exposed spots were isolated, and then subjected to secondary screening. Approximately 200 of the isolated colonies were displayed on each LB/Ampicillin plate, and colony lifting was performed in a similar manner to primary screening, which was followed by hybridization. The conditions for hybridization were the same as the conditions for primary screening.

As a result, a single colony of E. coli that reacted strongly with the probe was isolated. Plasmids were collected from the colony and, when the contained sequence was determined, a novel sequence having the same sequence as the sequence discovered in Example 9 (SEQ ID NO: 5) was found (the sequence of Candida GWT1), which was presumed to be a C. albicans homologue.

/51

Example 17: The S. Pombe Homologue of the S. cerevisiae GWT1 Gene

S. Pombe genes which showed homology to the S. cerevisiae GWT1 gene (SEQ ID NO: 27, and the amino acid sequence of the gene product thereof: SEQ ID NO: 28) were discovered from a database search, and were considered to be the S. Pombe homologues of GWT1.

Example 18: Cloning of the Aspergillus Fumigatus Homologue of the S. cerevisiae GWT1 Gene

By genetic sequence analysis, the inventors discovered two highly conserved regions in the protein encoded by the GWT1 genes of S. cerevisiae, S. pombe, and C. albicans (see, FIG. 7). Based on the presumed DNA that encodes the amino acid sequence of this conserved region, primers of SEQ ID NO: 29, SEQ ID NO: 30, and SEQ ID NO: 31 were designed. A PCR amplification was carried out using 1 μL of the library purchased from STRATAGENE (Aspergillus fumigatus cDNA library: #937053) as a template and using primers of SEQ ID NO: 29 and SEQ ID NO: 31. Further, as a result of carrying out nested-PCR using 1 μL of the resulting amplified sample as a template, and using primers of SEQ ID NO: 29 and SEQ ID NO: 30, the amplification of approximately 250 bp of a single fragment was confirmed. When the sequence of this fragment was determined, a novel sequence having homology to the GWT1 gene of S. cerevisiae, shown in SEQ ID NO: 32, was obtained, which was presumed to be the homologue of A. fumigatus.

In order to obtain a full length of cDNA, primers of SEQ ID NO: 33 and SEQ ID NO: 34 were designed based on the sequence of the amplified fragment. Further, primers outside the gene insertion site of the library, SEQ ID NO: 35 and SEQ ID NO: 36, were designed. As a result of performing PCR using the A. fumigatus cDNA library as a template, and the primer set of SEQ ID NO: 33 and SEQ ID NO: 35, or the primer set of SEQ ID NO: 34 and SEQ ID NO: 36, the amplification of approximately 1 kb of a DNA fragment was confirmed by both primer sets. As a result of determining the nucleotide sequences of these fragments, a novel sequence that was highly homologous to the GWT1 genes of S. cerevisiae shown in SEQ ID NO: 1 was obtained. Since the sequence is highly homologous to the GWT1 genes of S. cerevisiae, S. pombe, and C. albicans throughout the entire gene, this sequence was strongly suggested to be a homologue of A. fumigatus.

/52

In order to clone the entire homologue of A. fumigatus, the primer shown in SEQ ID NO: 37 that corresponded to the sequence upstream of the initiation codon, and the primer of SEQ ID NO: 38 that corresponded to the sequence downstream of the stop codon were newly designed based on the obtained sequence. As a result of performing 35 cycles of PCR using the A. fumigatus cDNA library (STRATAGENE) and the A. fumigatus genomic library (STRATAGENE) as templates, and primers of SEQ ID NO: 37 and SEQ ID NO: 38, 1.6 kb of a single amplified fragment was detected from both templates. As a result of determining the nucleotide sequence of this fragment by Direct-Sequencing, the nucleotide sequence shown in SEQ ID NO: 39 was discovered from the cDNA library, and was suggested to encode a protein comprising 501 amino acids shown in SEQ ID NO: 40. Further, the nucleotide sequence of SEQ ID NO: 41 was discovered from the genomic library, and was found to have an intron comprising 77 base pairs.

Example 19: Cloning of the Cryptococcus Homologue of the S. cerevisiae GWT1 Gene

1) Database Search

As a result of a database search for genes showing homology to the S. cerevisiae GWT1 gene, the sequence of 502042C05.xl was found from the server of the Genome Center at Stanford University (http://baggage.stanford.edu/cgi-misc/cneoformans/). Further, the sequence of b6e06cn.fl was found from the server at Oklahoma University, U.S.A (http://www.genome.ou.edu/cneo_blast.html).

/53

2) PCR Using Genomic DNA as Template

The primer of SEQ ID NO: 42 was constructed based on the sequence of 502042C05.xl, and the primer of SEQ ID NO: 43 was constructed based on the sequence of b6e06cn.fl. When PCR amplification with the primer of SEQ ID NO:42 and the primer of SEQ ID NO: 43 was carried out using the genomic DNA of Cryptococcus (Cryptococcus neoformans) as a template, approximately 2 kb of an amplified fragment was detected. When the nucleotide sequence of the resulting fragment was determined, a novel sequence showing homology to the GWT1 gene of S. cerevisiae, shown in SEQ ID NO: 44, was obtained.

In order to obtain the sequence upstream of the initiation codon of the Cryptococcus GWT1 gene, the primer of SEQ ID NO: 45 was designed based on the sequence of 502042C05.xl, and the primer of SEQ ID NO: 46 was designed based on the sequence of SEQ ID NO: 44. When a PCR amplification with the primer of SEQ ID NO:45, and the primer of SEQ ID NO:46 was carried out using the genomic DNA of Cryptococcus as a template, approximately 500 bp of an amplified fragment was detected. When the nucleotide sequence of the resulting fragment was determined, the sequence of SEQ ID NO: 47 was obtained, which was found to overlap with SEQ ID NO: 44.

3) 3'-RACE

3'-RACE was carried out in order to obtain the 3'-terminal sequence of the Cryptococcus GWT1 gene. A reverse transcription was carried out by priming with the adaptor-primer of SEQ ID NO: 48, which was based on 16 μg of total RNA extracted from Cryptococcus, and by using SuperScript II Reverse Transcriptase (GIBCO/BRL), so that a single stranded cDNA, which was to become the template for the RT-PCR later, was produced. As a result of performing 35 cycles of PCR with the primers of SEQ ID NO: 49 and SEQ ID NO: 50 using the single stranded cDNA as a template, approximately 1.2 kb of an amplified fragment was detected. When the nucleotide sequence of the resulting fragment was analyzed by the Direct-Sequencing method, the novel sequence shown in SEQ ID NO: 51 showing homology to the S. cerevisiae GWT1 gene was obtained.

4) PCR of a Full Length Genomic DNA

/54

Using the primer of SEQ ID NO: 52 that was designed based on SEQ ID NO: 47, and the primer of SEQ ID NO: 53 that was designed based on SEQ ID NO: 51, 35 cycles of PCR was carried out on three independent preparations with the genomic DNA of Cryptococcus as a template. As a result, approximately 2 kb of an amplified fragment was detected from all three of the independent tubes, and each of them was therefore individually subjected to Direct-Sequencing in order to determine the entire nucleotide sequences respectively. As a result, the three independent sequences completely matched and a sequence comprising the full length of GWT1 gene homologue of Cryptococcus shown in SEQ ID NO: 54 was obtained.

5) Determination of the cDNA Sequence

The comparison of the sequence of the Cryptococcus GWT1 gene derived from the genome shown in SEQ ID NO: 54 with the cDNA sequence (SEQ ID NO: 51) obtained by 3'-RACE suggested the presence of introns at two positions. Further, since the open reading frame following the ATG initiation codon was not continuous, the presence of another intron was also suggested. Therefore, the cDNA structure was predicted from the presumed amino acid sequence and the splicing donor/acceptor sequence, and the primers of SEQ ID NO: 55 and SEQ ID NO: 56 were designed to be a junction at the position predicted between exons. As a result of performing 35 cycles of PCR using the single stranded cDNA derived from Cryptococcus as a template with the above-mentioned primers, approximately 1.4 kb of an amplified fragment was confirmed. As a result of determining the nucleotide sequence by subjecting the resulting fragment to Direct-Sequencing, the sequence of SEQ ID NO: 57 was obtained, and by comparing with SEQ ID NO: 54, the cDNA sequence of the GWT1 gene of Cryptococcus was suggested to have the structure of SEQ ID NO: 58. Since the sequence shows high homology at certain regions with the GWT1 genes of S. cerevisiae, S. pombe, C. albicans, and A. fumigatus, the sequence was strongly suggested to be a homologue of Cryptococcus.

Example 20: Preparation of Membrane Fraction Expressing the GWT1 Protein

(1) Preparation of GWT1 expressing vector

/55

In order to prepare the expression vector in S. *cerevisiae*, YEp352GAPII vector was prepared by inserting a GAPDH promoter and a GAPDH terminator, both derived from pKT10 (Tanaka et al., Mol. Cell Biol., 10: 4303-4313, 1990), into the multi-cloning site of YEp352; and replacing the multi-cloning site with that of pUC18. Further, in order to facilitate the insertion of the GWT1 gene, YEp352GAPIIClaIδSal vector was prepared by substituting the ClaI site for the SalI site in the multi-cloning site.

The S. cerevisiae GWT1 gene comprising the nucleotide sequence of SEQ ID NO: 1 was amplified using primers of SEQ ID NO: 64 and SEQ ID NO: 65 and then inserted into the

multi-cloning site of YEp352GAPIIClaIδSal vector, so that a GWT1-overexpressing plasmid was prepared.

(2) Preparation of the membrane fraction

S. cerevisiae G2-10 strain was cultured by shaking in 10 mL of YPD medium at a temperature of 30 degrees Celsius, and then the cells were collected at the late logarithmic growth phase (OD_{600}=2-5). After washing with sterilized water, the above-mentioned plasmid and an empty vector containing no GWT1 were introduced into the S. cerevisiae G2-10 strain by lithium acetate method using YEASTMAKER™ Yeast Transformation System (Clontech) (according to the YEASTMAKER™ Yeast Transformation System User Manual). A GWT1 or mutated-GWT1-overexpressing strain and an empty vector-introduced strain were be obtained by culturing the cells in SD(ura^-) medium at a temperature of 30 degrees Celsius for two days. Each resulting strain was cultured by shaking in SD(ura^-) liquid medium at a temperature of 30 degrees Celsius, and then the cells were collected at the middle logarithmic growth phase (OD_{600}=1-3). After being washed with 10 mM NaN_3, the resulting cells were suspended in three times the amount of homogenization buffer cells (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, Complete™; manufactured by Roche), and four times the amount of glass bead cells was added to the suspension. The resulting samples were vortexed and placed on ice; and these procedures were repeated four times in order to disrupt fungal cells. 1 mL of homogenization buffer was added to the resulting sample and the samples were then centrifuged at a rotational speed of 2,500 rpm for five minutes at a temperature of four degrees Celsius in order to precipitate glass beads and fungal cells which had not been disrupted. The resulting supernatant was taken to another tube and then centrifuged at a rotational speed of 135,000 rpm for 10 minutes at a temperature of four degrees Celsius in order to precipitate the membrane fraction containing organelles (total membrane fraction). The precipitate was further suspended in 1 mL of a binding buffer (0.1 M Phosphate buffer, pH 7.0, 0.05%

/56

Tween 20, Complete™, manufactured by Roche) and then centrifuged at a rotational speed of 2,500 rpm for one minute at a temperature of four degrees Celsius in order to remove the components which were not suspended, and the supernatant was then centrifuged at a rotational speed of 15,000 rpm for five minutes at a temperature of five degrees Celsius. The precipitate was further suspended in

150 to 650 μL of binding buffer in order to obtain a membrane fraction.

Example 21: Binding assay of labeled compound

(1) Synthesis of labeled compound

10 mg (0.036 mmol) of the compound of Example 2 (Compound B2), 20 mg of palladium oxide and 10 mg of palladium-carbon (10%) were dissolved in 1 mL of methanol and then stirred under 10 Ci of tritium gas for 50 minutes. Subsequently, the reaction solution was filtrated and the filtrate was concentrated to dryness. In order to remove active tritium, the filtrate was dissolved in methanol and then concentrated to dryness three times. The residue was fractionated on a high-performance liquid chromatography. The peak fractions were collected, concentrated to dryness and then dissolved in ethanol.

(2) Binding assay

1 ng of [^{3}H] compound B2 was added to the prepared membrane fraction and the mixture was then allowed to stand on ice for one to two hours in order to carry out the binding reaction between the [^{3}H] compound B2 and the membrane fraction. The membrane fraction was precipitated by centrifuging the mixture at a rotational speed of 15,000 rpm for three minutes at a temperature of four degrees Celsius. The operation in which the precipitate was suspended in 150 μL of binding buffer and the suspension was centrifuged at a rotational speed of 15,000 rpm for three minutes at a temperature of four degrees Celsius was repeated twice in order to remove any unbound [^{3}H] compound B2. The precipitate was again suspended in 80 μL of binding buffer and the resulting suspension was transferred into a scintillation vial, and 6 mL of a scintillator was added thereto in order to measure the radioactivity using a liquid scintillation counter (Aloka).

As a result, as shown in FIG. 8, while the [^{3}H] compound B2 binding was not observed very much in the membrane fraction of the strain into which a vector containing no GWT1 gene was introduced, the binding amount of the strain overexpressing GWT1 significantly increased.

/57

Further, the increased binding was completely inhibited by adding 100 volumes of cold compound B2. As described above,

it was suggested that [3H] compound B2 specifically binds to the GWT1 proteins in the membrane fraction.

Example 22: Binding inhibition of [^{3}H] compound B2 by a test sample

Screening the compound which inhibited the binding of [^{3}H] compound B2 and GWT1 was attempted using the present assay system. 100 ng of a test sample (the compound of Example 32: compound B32, or the compound of Example 16: compound B16) and 1 ng of [^{3}H] compound B2 were added to 100 µL of the prepared membrane fraction and the mixture was then allowed to stand on ice for one to two hours in order to carry out the binding reaction of the test sample, the [^{3}H] compound B2 and the membrane fraction, and the mixture was then washed in order to measure the radioactivity according to the method in Example 21.

As shown in FIG. 9, the binding of [^{3}H] compound B2 to the membrane fraction was inhibited in a concentration-dependent manner by adding one, 10 and 100 volumes of unlabeled compound B2. Further, the binding was also inhibited by adding 100 volumes of an analog Compound B32 (IC_{50} = 0.78 µg/mL) which had the same activity as compound B2 in the reporter system described in Example 2. By contrast, the binding was not inhibited by adding 100 volumes of an analog Compound B16 (IC_{50} > 200 µg/mL) which showed no activity in the reporter system described in Example 2 As described above, the present assay system can be used in order to select a compound which binds to GWT1 proteins.

Example 23: Binding Assay of Labeled Compound to Candida Albicans GWT1 Gene Product

(1) Preparation of candida albicans GWT1 expressing vector

The candida albicans GWT1 gene product ("CaGWT1", comprising the nucleotide sequence of SEQ ID NO: 3 and SEQ /58
ID NO: 5) was amplified using primers of SEQ ID NO: 66 and SEQ ID NO: 67 and then inserted into the multi-cloning site of YWp352GAP11 vector, so that a CaGWT1-overexpressing plasmid was prepared.

(2) Preparation of the membrane fraction

The CaGWT1-overexpressing plasmid and GWT1-expressing plasmid were introduced into the S. cerevisiae G2-10 strain according to the method in Example 20, so that CaGWT1 or GWT1-overexpressing strain was obtained. The membrane fraction was prepared by using the strain obtained according to the method in Example 20.

(3) Binding assay

A binding assay of the membrane fraction to [^{3}H] compound 1 was carried out according to the method in Example 21.

As shown in FIG 10, in the membrane fraction containing the strain overexpressing CaGWT1, the binding amount significantly increased similarly to the strain overexpressing GWT1. Further, the increased binding was completely inhibited by adding 100 volumes of cold compound 1. As described above, [3H] compound 1 specifically binds to the CaGWT1 protein in the membrane fraction, and it was suggested that the present binding assay could be used in order to select a compound which bound to CaGWT1 protein.

Example 24

Compounds were evaluated using the S. cerevisiae reporter system described in Example 2. The lowest concentration at which cephalosporinase activity in the cell wall fraction became 50% or less compared to that obtained where the compound was not treated, was defined to be the IC 50 value. The effects of the typical compounds are shown in Table 1.

/59

Table 1

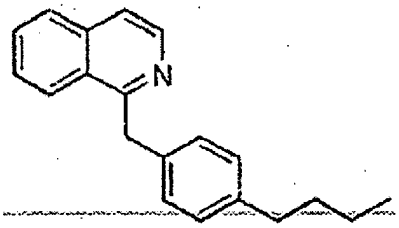
Compounds	IC50 (μg/mL)
1-(4-butylbenzyl)isoquinoline (Compound 1)	0.39
N-{3-[4-(1-isoquinolylmethyl)phenyl]-2-propynyl}acetamide (Compound 40)	6.25
N-{3-[4-(1-isoquinolylmethyl)phenyl]-propyl}-N-methylacetamide (Compound 50)	50
5-butyl-2-(1-isoquinolylmethyl)phenol (Compound 56)	0.20
4-(4-butylbenzyl)thieno[3,2-c]pyridine (Compound 134)	0.78
7-(4-butylbenzyl)thieno[2.3-c]pyridine (Compound 140)	0.39
2-(4-butylbenzyl)-3-methoxypyridine (Compound 159)	0.78
2-(4-butylbenzyl)-3,4-dimethoxypyridine (Compound 166)	0.78

Refer to Japanese Patent Application No. 2001-401947 for the compounds used in the evaluation in the present examples and their synthesis methods. The typical compounds thereof are shown below:

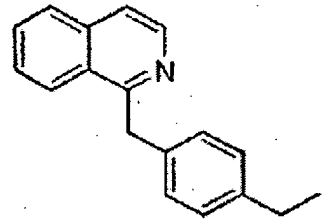
/60

[Compounds List]

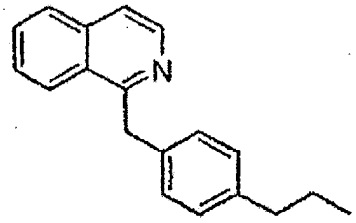
1. 1-(4-butylbenzyl)isoquinoline



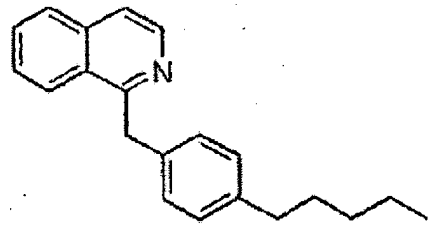
2. 1-(4-ethylbenzyl)isoquinoline



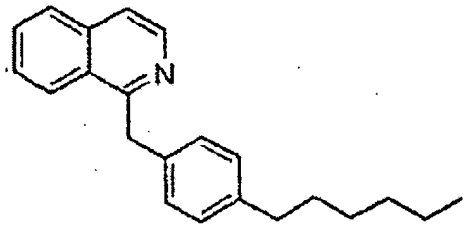
3. 1-(4-propylbenzyl)isoquinoline



4. 1-(4-pentylbenzyl)isoquinoline

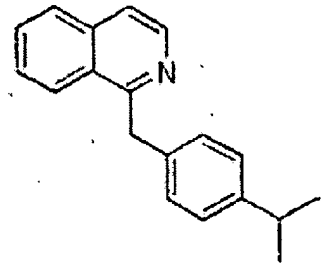


5. 1-(4-hexylbenzyl)-isoquinoline

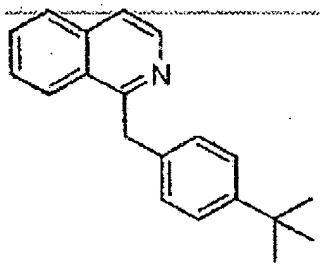


/61

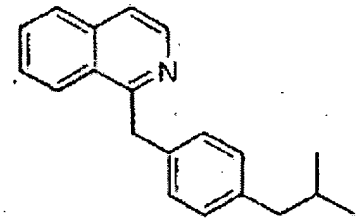
6. 1-(4-isopropylbenzyl)isoquinoline



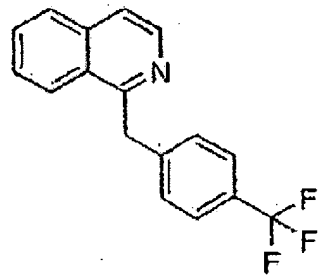
7. 1-[4-(tert-butyl)benzyl]isoquinoline



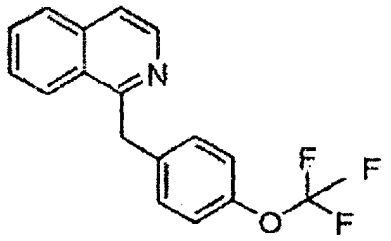
8. 1-(4-isobutylbenzyl)isoquinoline



9. 1-[4-(trifluoromethyl)benzyl]isoquinoline

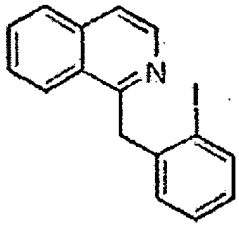


10. 1-[4-(trifluoromethoxy)benzyl]isoquinoline

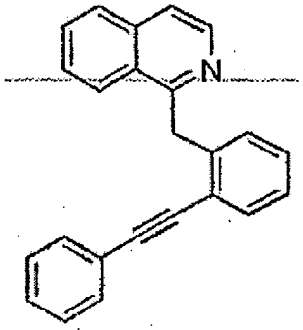


/62

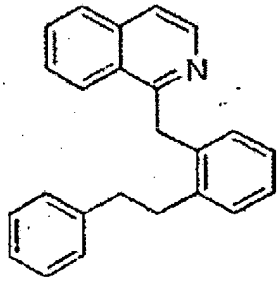
11. 1-(2-iodobenzyl)isoquinoline



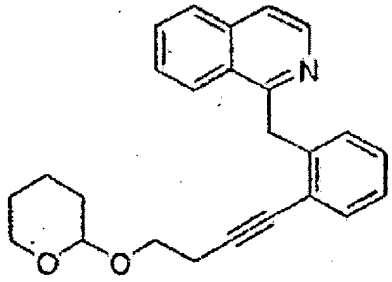
12. 1-[2-(2-phenyl-1-ethynyl)benzyl]isoquinoline



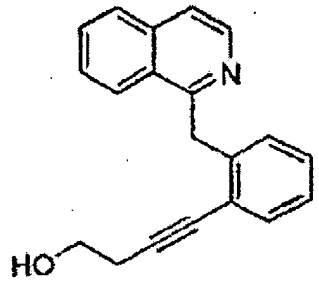
13. 1-(2-phenylethylbenzyl)isoquinoline



14. 1-{2-[4-(tetrahydro-2H-2-pyranyloxy)-1-butynyl]benzyl}isoquinoline

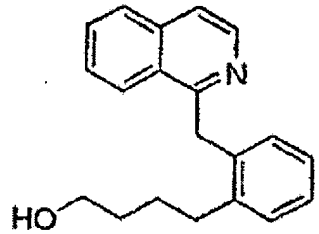


15. 4-[2-(1-isoquinolylmethyl)phenyl]-3-butyn-1-ol

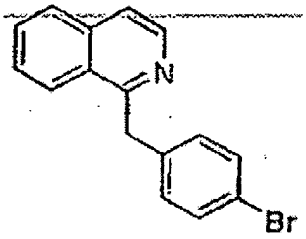


/63

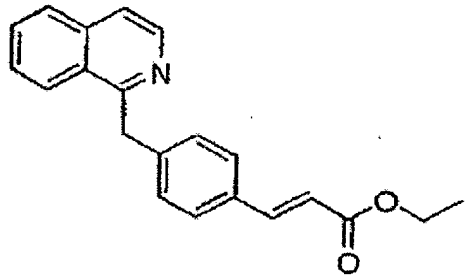
16. 4-[2-(1-isoquinolylmethyl)phenyl]-1-butanol



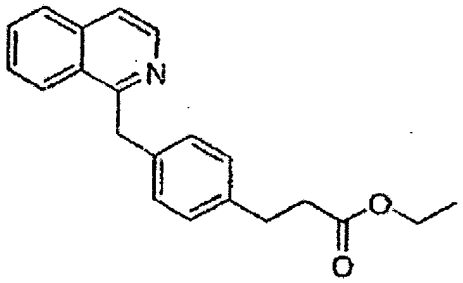
17. 1-(4-bromobenzyl)isoquinoline



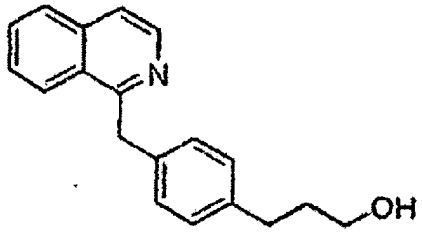
18. Ethyl(E)-3-[4-(isoquinolylmethyl)phenyl]-2-propenoate



19. Ethyl-3-[4-(isoquinolylmethyl)phenyl]-2-propanoate

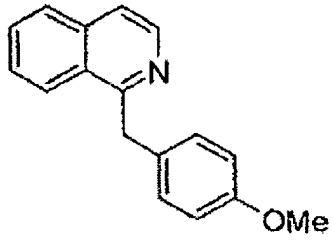


20. 3-[4-(1-isoquinolylmethyl)phenyl]-1-propanol

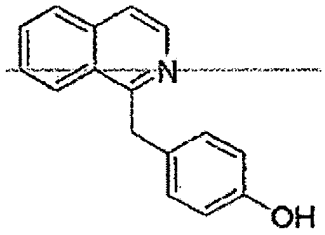


/64

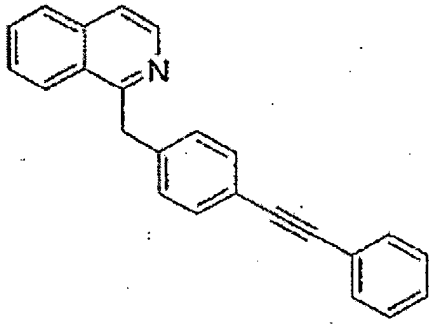
21. 1-(4-methoxybenzyl)isoquinoline



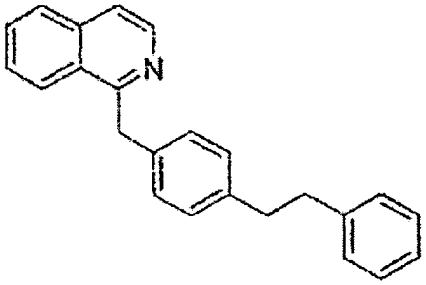
22. 4-(1-isoquinolylmethyl)phenol



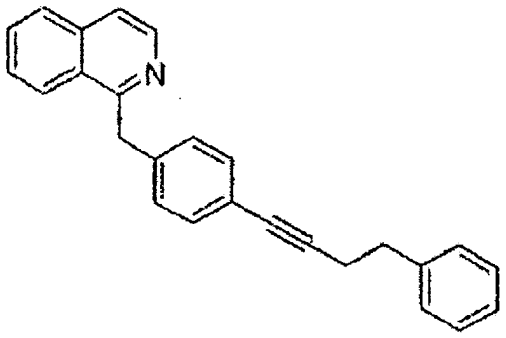
23. 1-[4-(2-phenyl-1-ethynyl)benzyl]isoquinoline



24. 1-(4-phenethylbenzyl)isoquinoline



25. 1-[4-(4-phenyl-1-butynyl)benzyl]isoquinoline



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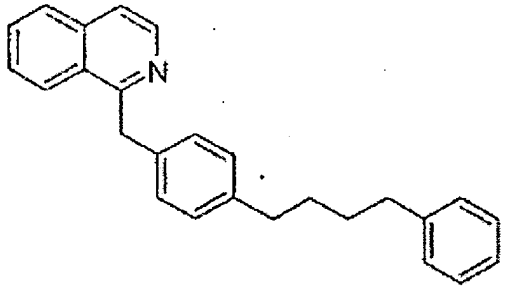
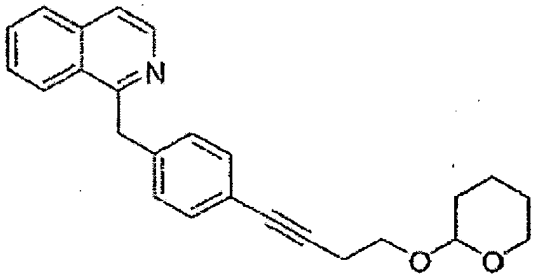
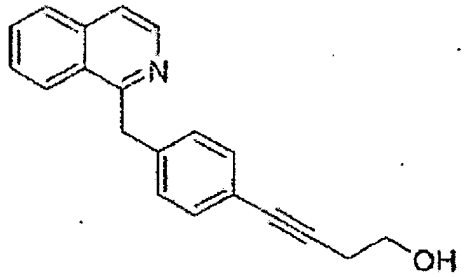
26. 1-[4-(4-phenyl-1-butyl)benzyl]isoquinoline



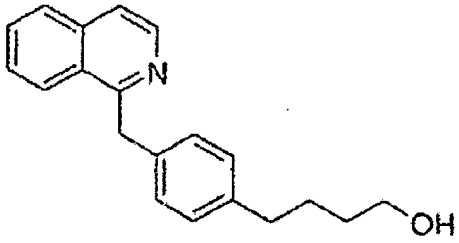
27. 1-{4-[4-(tetrahydro-2H-2-pyranyloxy)-1-butynyl]benzyl}isoquinoline



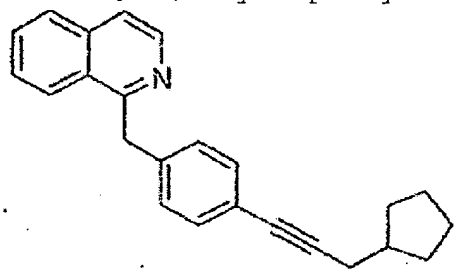
28. 4-[4-(1-isoquinolylmethyl)phenyl]-3-butyn-1-ol



29. 4-[4-(1-isoquinolylmethyl)phenyl]-1-butanol



30. 1-[4-(3-cyclopentyl-1-propenyl)benzyl]isoquinoline



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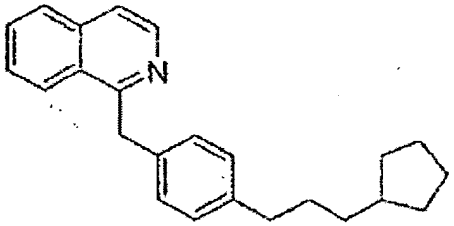
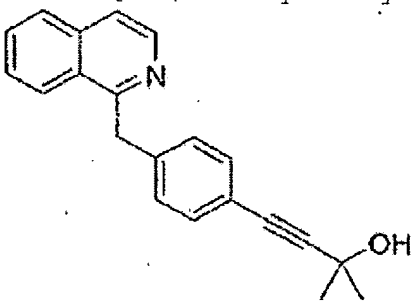
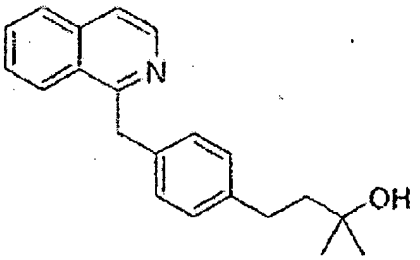
31. 1-[4-(3-cyclopentylpropyl)benzyl]isoquinoline



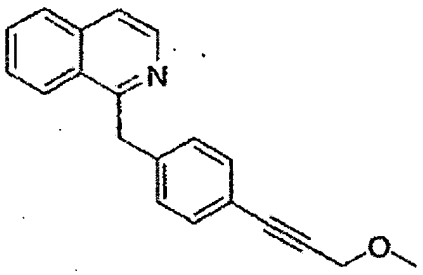
32. 4-[4-(1-isoquinolylmethyl)phenyl]-2-methyl-3-butyn-2-ol



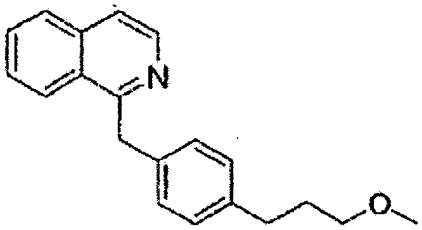
33. 4-[4-(1-isoquinolylmethyl)phenyl]-2-methyl-2-butanol



34. 1-[4-(3-methoxy-1-propynyl)benzyl]isoquinoline



35. 1-[4-(3-methoxypropyl)benzyl]isoquinoline



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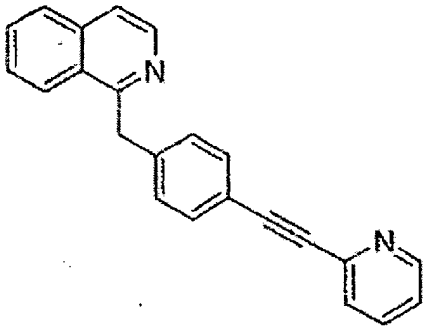
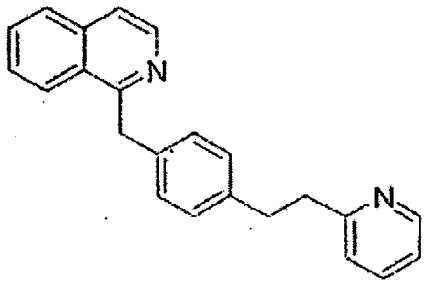
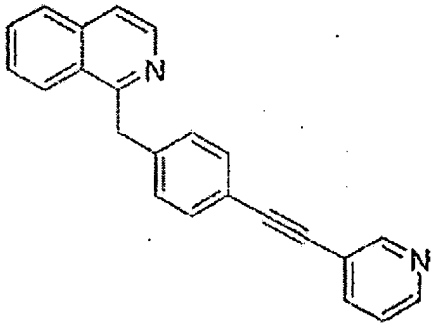
36. 1-{4-[2-(2-pyridyl)-1-ethynyl]benzyl}isoquinoline



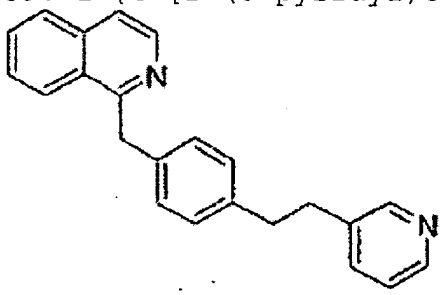
37. 1-{4-[2-(2-pyridyl)ethyl]benzyl}isoquinoline



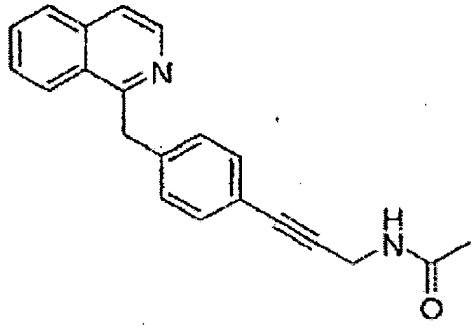
38. 1-{4-[2-(3-pyridyl)-1-ethynyl]benzyl}isoquinoline



39. 1-{4-[2-(3-pyridyl)ethyl]benzyl}isoquinoline

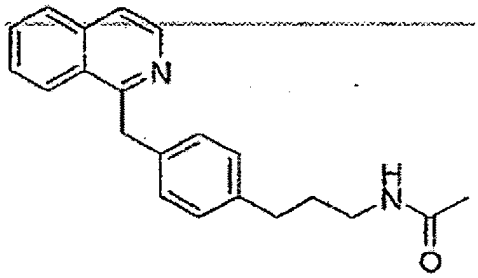


40. N-{3-[4-(1-isoquinolylmethyl)phenyl]-2-propynyl}acetamide

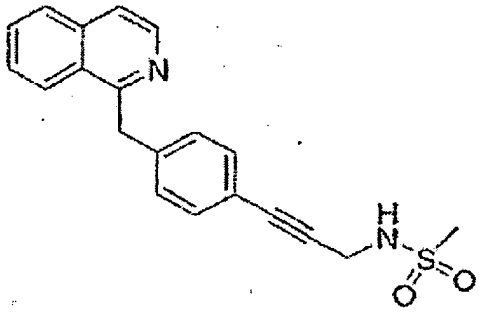


/68

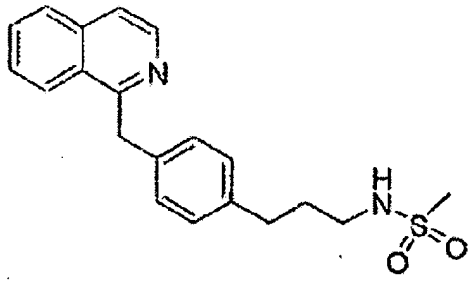
41. N-{3-[4-(1-isoquinolylmethyl)phenyl]propyl}acetamide



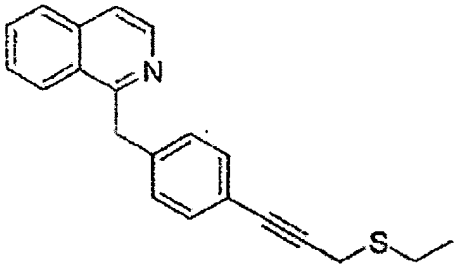
42. N-{3-[4-(1-isoquinolylmethyl)phenyl]-2-propynyl}methanesulfonamide



43. N-{3-[4-(1-isoquinolylmethyl)phenyl]propyl}methanesulfonamide

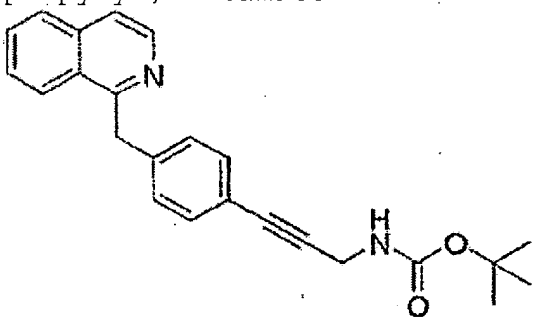


44. 1-{4-[3-(ethylsulfanyl)-1-propynyl]benzyl}isoquinoline

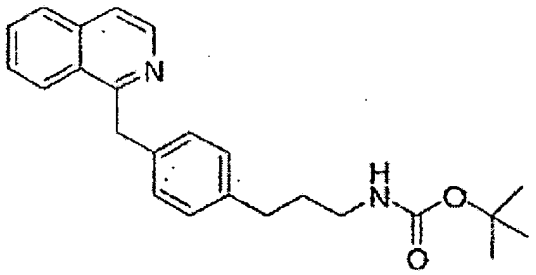


/69

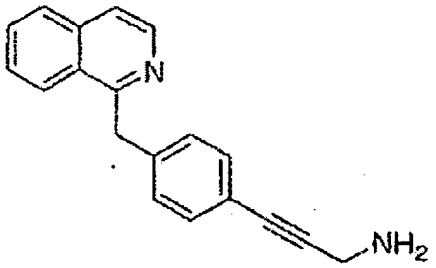
45. tert-butyl N-{3-[4-(1-isoquinolylmethyl)phenyl]-2-propynyl}carbamate



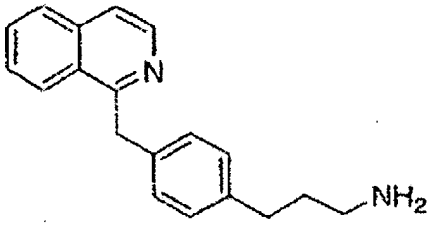
46. tert-butyl N-{3-[4-(1-isoquinolylmethyl)phenyl]propyl}carbamate



47. 3-[4-(1-isoquinolylmethyl)phenyl]-2-propyn-1-yl-amine



48. 3-[4-(1-isoquinolylmethyl)phenyl]-1-propanamine



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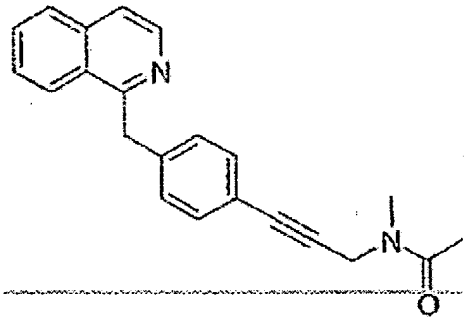
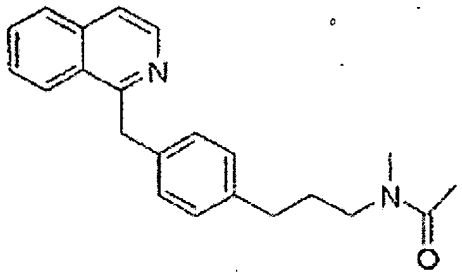
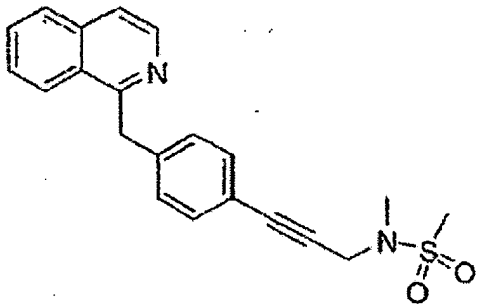
49. N-{3-[4-(1-isoquinolylmethyl)phenyl]-2-propynyl}-N-methylacetamide



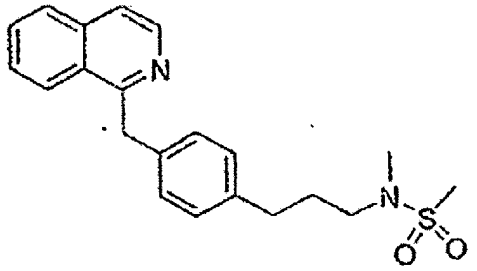
50. N-{3-[4-(1-isoquinolylmethyl)phenyl]propyl}-N-methylacetamide



51. N-{3-[4-(1-isoquinolylmethyl)phenyl]-2-propynyl}-N-methyl methanesulfonamide



52. N-{3-[4-(1-isoquinolylmethyl)phenyl]propyl}-N-methyl methanesulfonamide



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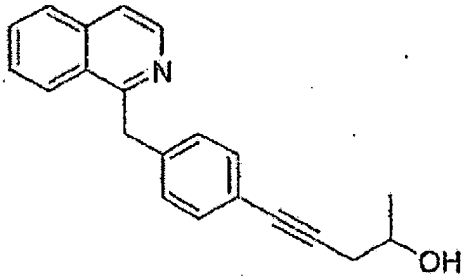
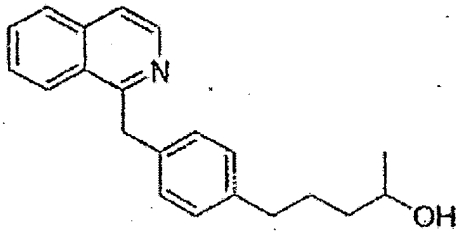
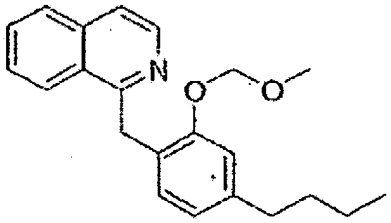
53. 5-[4-(1-isoquinolylmethyl)phenyl]-4-pentyn-2-ol



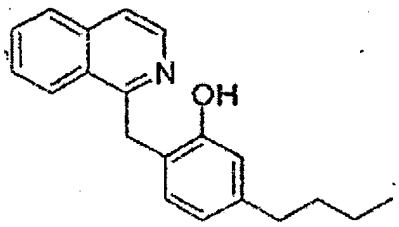
54. 5-[4-(1-isoquinolylmethyl)phenyl]-2-pentanol



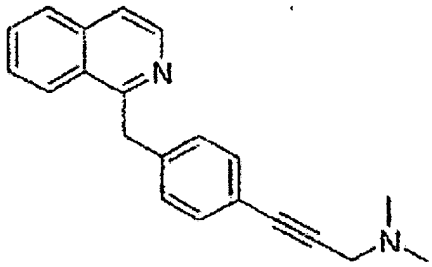
55. 1-[4-butyl-2-(methoxymethoxy)benzyl]isoquinoline



56. 5-butyl-2-(1-isoquinolylmethyl)phenol

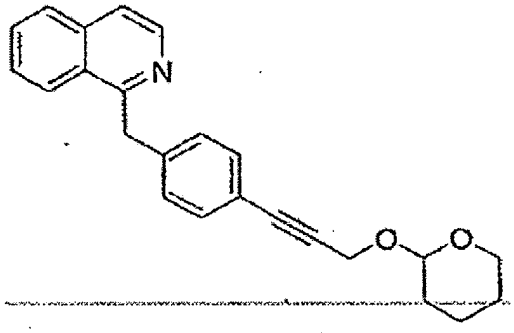


57. N-{3-[4-(1-isoquinolylmethyl)phenyl]-2-propynyl}-N,N-dimethylamine

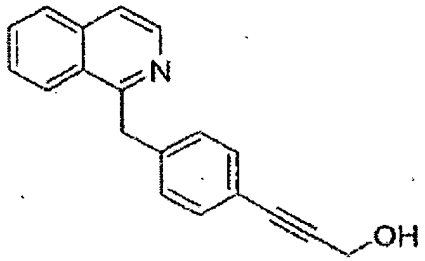


/72

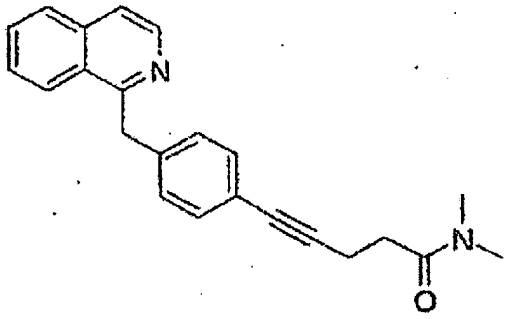
58. 1-{4-[3-(tetrahydro-2H-2-pyranyloxy)-1-propynyl]benzyl}isoquinoline



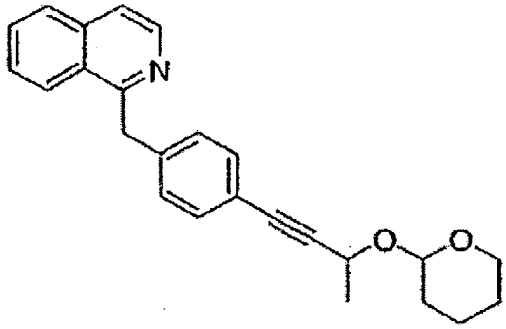
59. 3-[4-(1-isoquinolylmethyl)phenyl]-2-propyn-1-ol



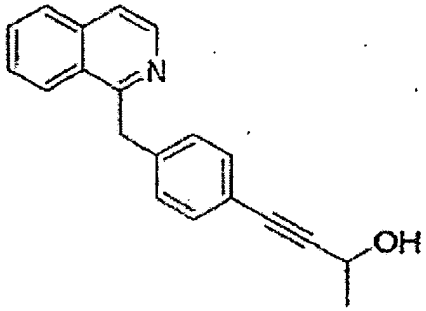
60. N,N-dimethyl-5-[4-(1-isoquinolylmethyl)phenyl]-4-pentynamide



61. 1-{4-[3-(tetrahydro-2H-2-pyranyloxy)-1-butynyl]benzyl}isoquinoline

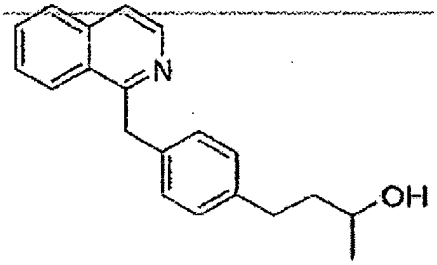


62. 4-[4-(1-isoquinolylmethyl)phenyl]-3-butyn-2-ol

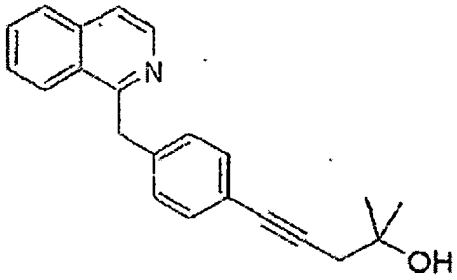


/73

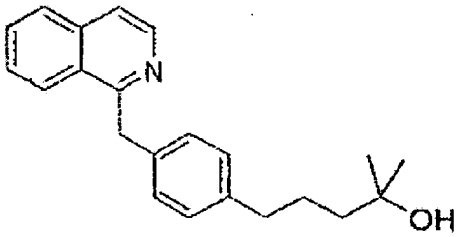
63. 4-[4-(1-isoquinolylmethyl)phenyl]-2-butanol



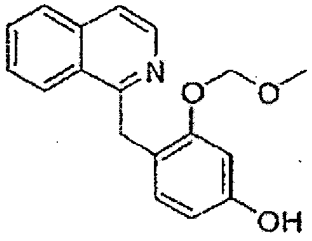
64. 5-[4-(1-isoquinolylmethyl)phenyl]-2-methyl-4-pentyn-2-ol



65. 5-[4-(1-isoquinolylmethyl)phenyl]-2-methyl-2-pentanol

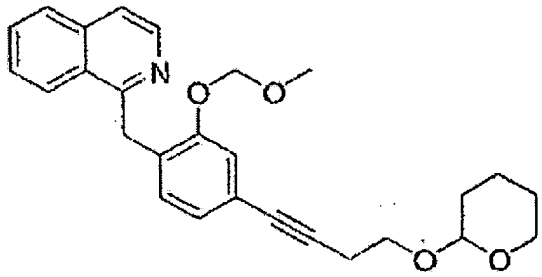


66. 4-(1-isoquinolylmethyl)-3-(methoxymethoxy)phenol

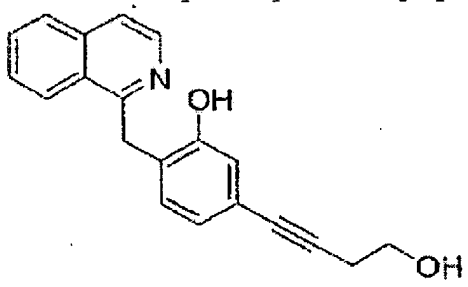


/74

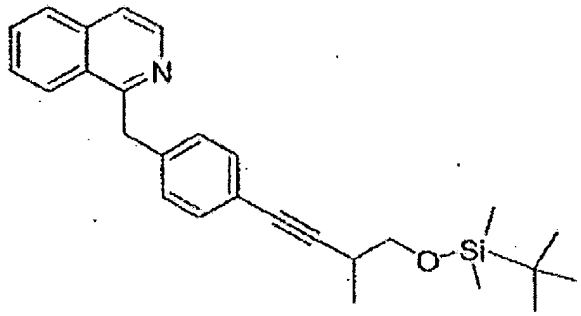
67. 1-{2-(methoxymethoxy)-[4-(tetrahydro-2H-2-pyranyloxy)-1-butynyl]benzyl}isoquinoline



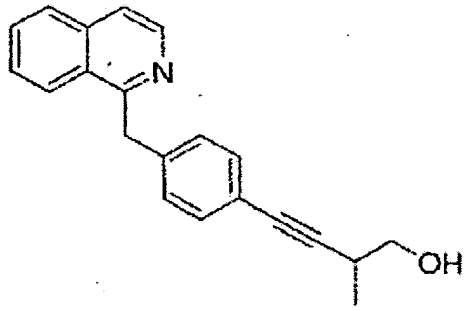
68. 5-(4-hydroxy-1-butynyl)-2-(1-isoquinolylmethyl)phenol



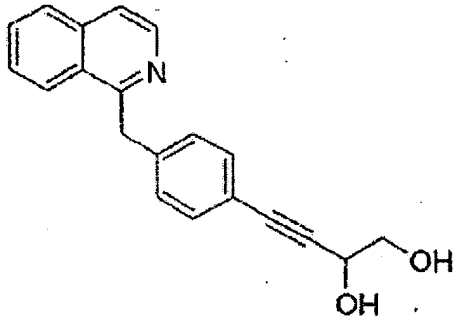
69. 1-(t-butyl)-1,1-dimethylsilyl {4-[4-(1-isoquinolylmethyl)phenyl]-2-methyl-3-butynyl}ether



70. 4-[4-(1-isoquinolylmethyl)phenyl]-2-methyl-3-butyn-1-ol

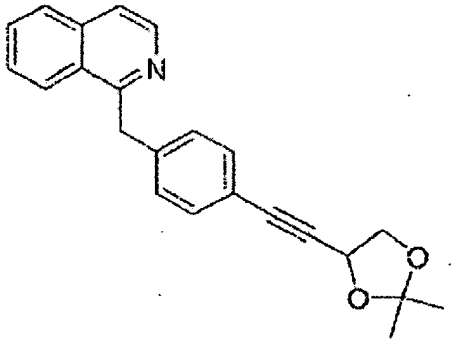


71. 4-[4-(1-isoquinolylmethyl)phenyl]-3-butyn-1,2-diol

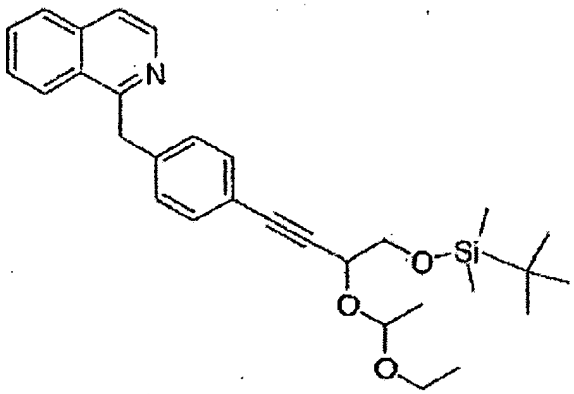


/75

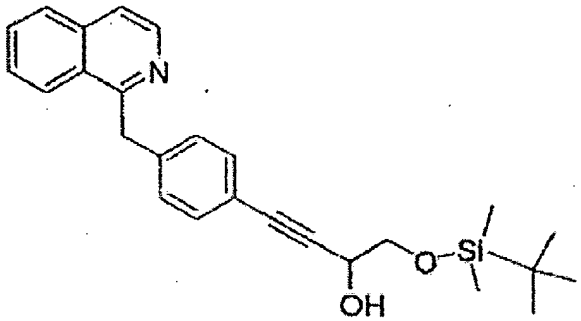
72. 1-{4-[2-(2,2-dimethyl^1,3-dioxyolan-4-yl)-1-ethynyl]benzyl}isoquinoline



73. 1-{4-[4-{[1-(t-butyl)-1,1-dimethylsilyl]oxy}-3-(1'-ethoxyethoxy)-1-butynyl]benzyl}isoquinoline

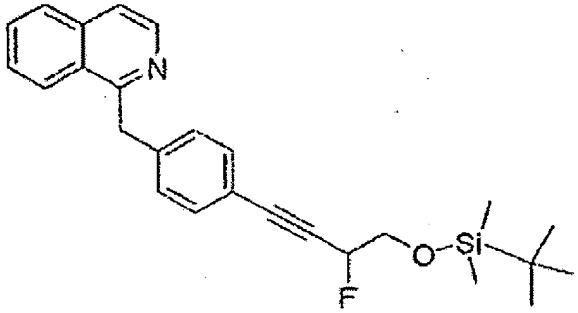


74. 1-{[1-(t-butyl)-1,1-dimethylsilyl]oxy}-4-[4-(1-isoquinolylethyl)phenyl]-3-butyn-2-ol

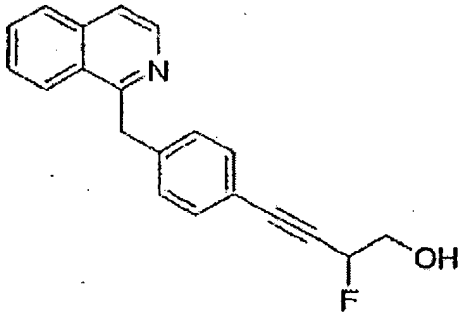


/76

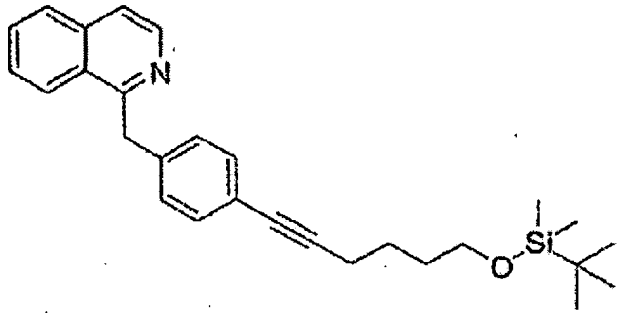
75. 1-(t-butyl)-1,1-dimethylsilyl {2-fluoro-4-[4-(1-isoquinolylmethyl)phenyl]-3-butynyl}ether



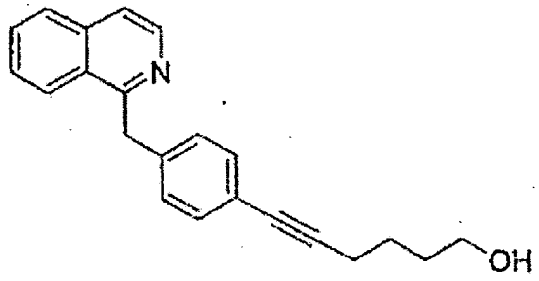
76. 2-[fluoro-4-[4-(1-isoquinolylmethyl)phenyl]-3-butyn-1-ol



77. 1-(t-butyl)-1,1-dimethylsilyl {6-[4-(1-isoquinolylmethyl)phenyl]-5-hexynyl}ether

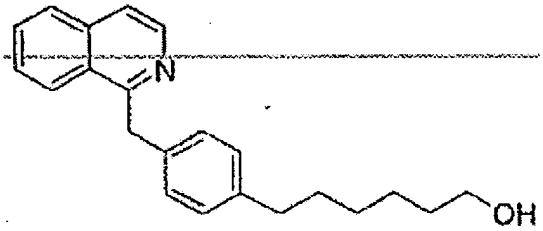


78. 6-[4-(1-isoquinolylmethyl)phenyl]-5-hexyn-1-ol

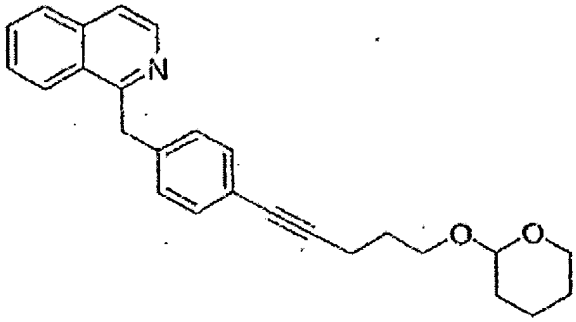


/77

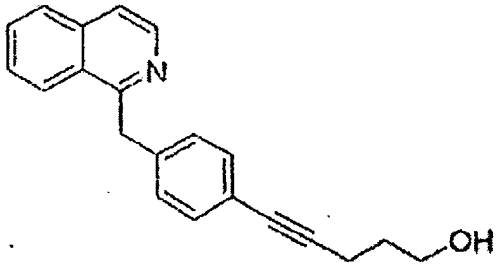
79. 6-[4-(1-isoquinolylmethyl)phenyl]-1-hexanol



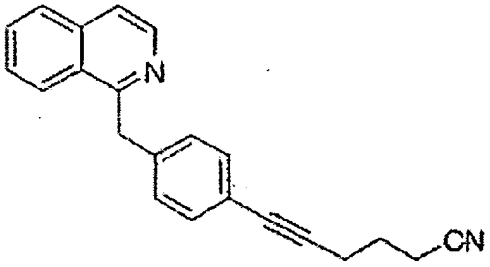
80. 1-{4-[5-(tetrahydro-2H-2-pyranyloxy)-1-pentynyl]benzyl}isoquinoline



81. 5-[4-(1-isoquinolylmethyl)phenyl]-4-pentyn-1-ol

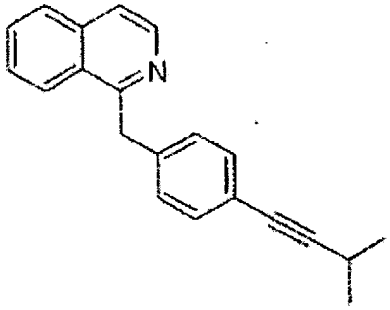


82. 5-[4-(1-isoquinolylmethyl)phenyl]-4-pentynylcyanide

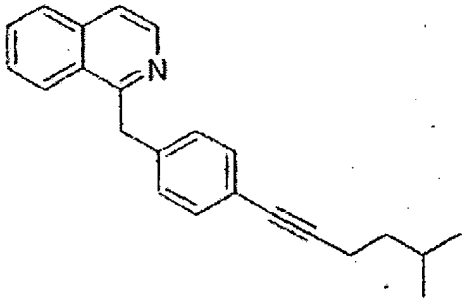


/78

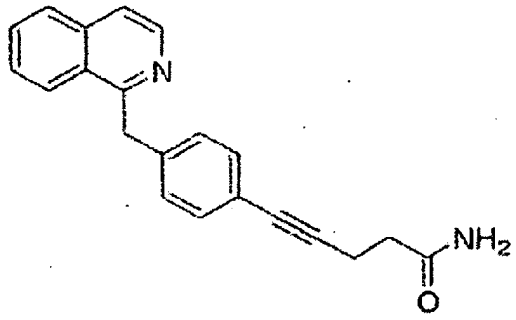
83. 1-[4-(3-methyl-1-butynyl)benzyl]isoquinoline



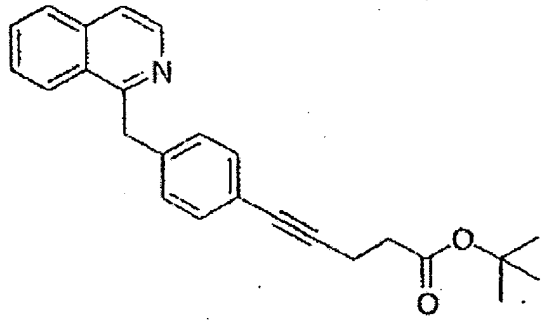
84. 1-[4-(5-methyl-1-hexynyl)benzyl]isoquinoline



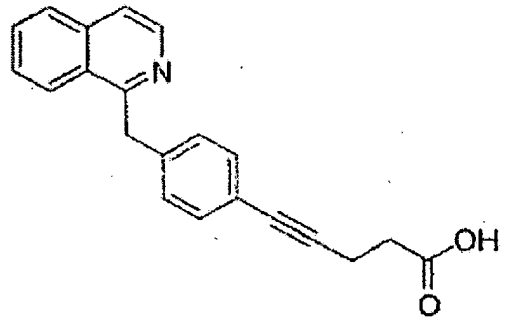
85. 5-[4-(1-isoquinolylmethyl)phenyl]-4-pentynamide



86. t-butyl 5-[4-(1-isoquinolylmethyl)phenyl]-4-pentynoate

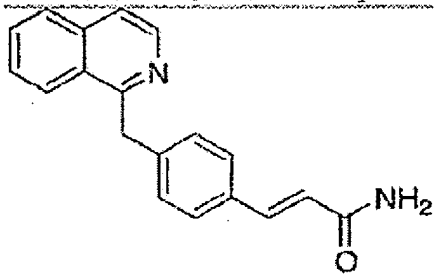


87. 5-[4-(1-isoquinolylmethyl)phenyl]-4-pentynoic acid

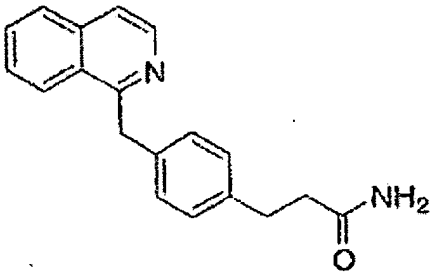


/79

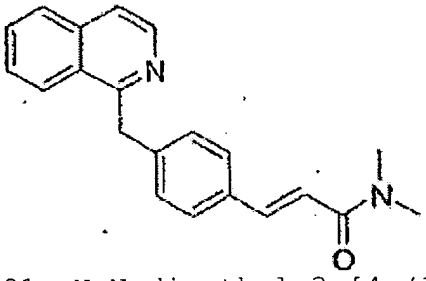
88. (E)-3-[4-(1-isoquinolylmethyl)phenyl]-2-propenamide



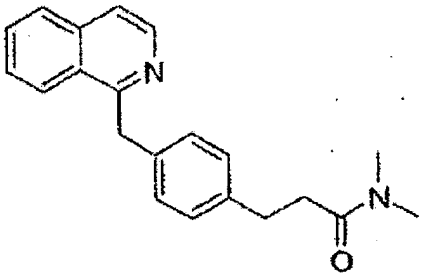
89. 3-[4-(1-isoquinolylmethyl)phenyl]-2-propanamide



90. N,N-dimethyl-(E)-3-[4-(1-isoquinolylmethyl)phenyl]-2-propenamide



91. N,N-dimethyl 3-[4-(1-isoquinolylmethyl)phenyl]propanamide



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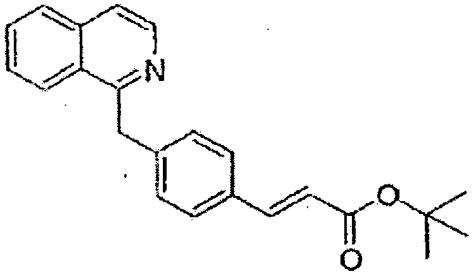
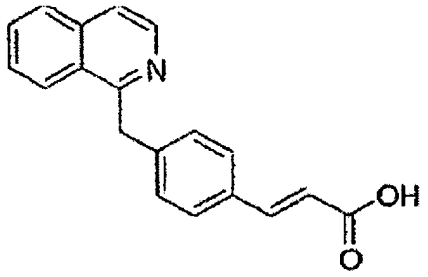
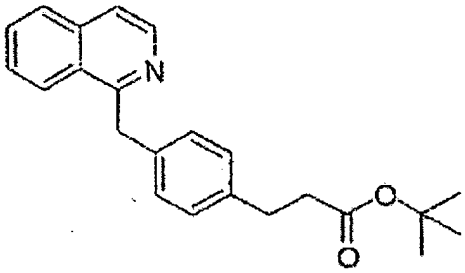
92. t-butyl (E)-3-[4-(1-isoquinolylmethyl)phenyl]-2-propenoate



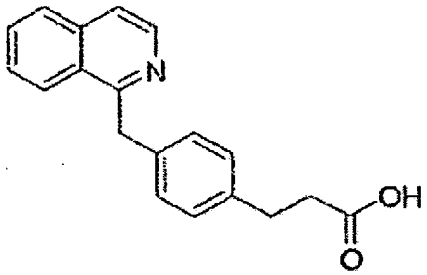
93. (E)-3-[4-(1-isoquinolylmethyl)phenyl]-2-propenoic acid



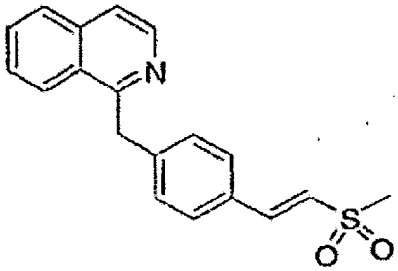
94. t-butyl 3-[4-(isoquinolylmethyl)phenyl]propanoate



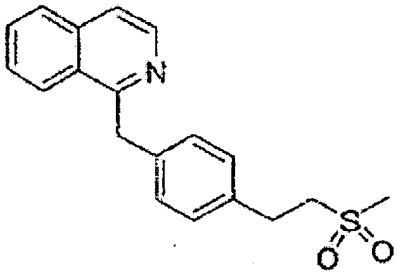
95. 3-[4-(1-isoquinolylmethyl)phenyl]propanoic acid



96. (E)-2-[4-(1-isoquinolylmethyl)phenyl]-1-ethenylmethylsulfone

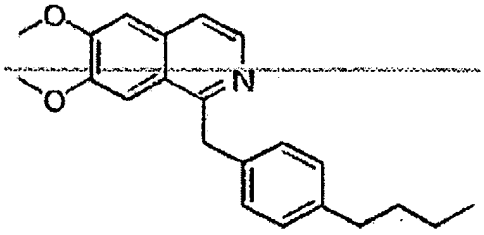


97. 1-{4-[2-(methylsulfonyl)ethyl]benzyl}isoquinoline

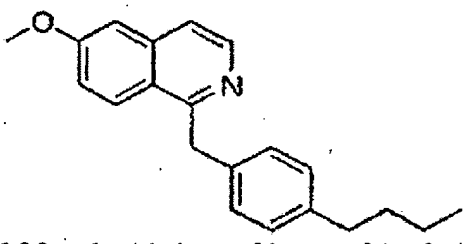


/81

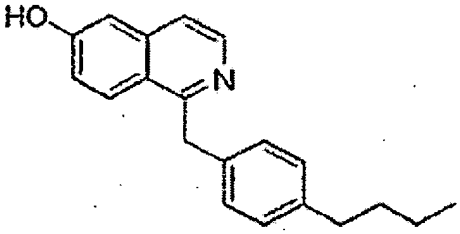
98. 1-(4-butylbenzyl)-6,7-dimethoxyisoquinoline



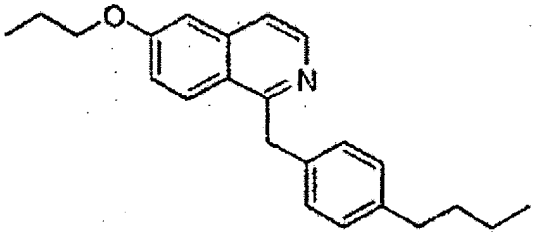
99. 1-(4-butylbenzyl)-6-methoxyisoquinoline



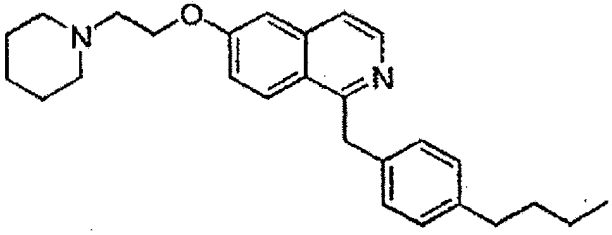
100. 1-(4-butylbenzyl)-6-isoquinolinol



101. 1-(4-butylbenzyl)-6-propoxyisoquinoline

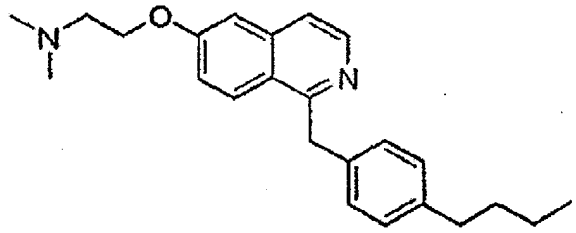


102. 1-(4-butylbenzyl)-6-(2-pyperidinoethoxy)isoquinoline

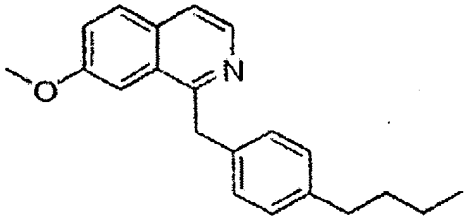


/82

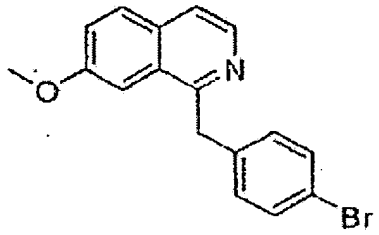
103. N-(2-{[1-(4-butylbenzyl)-6-isoquinolyl]oxy}ethyl)-N,N-dimethylamine



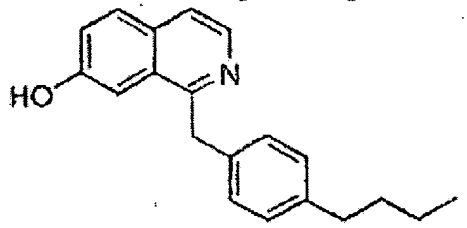
104. 1-(4-butylbenzyl)-7-methoxyisoquinoline



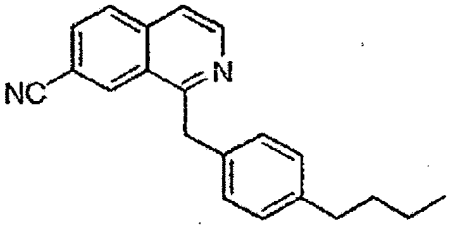
105. 1-(4-bromobenzyl)-7-methoxyisoquinoline



106. 1-(4-butylbenzyl)-7-isoquinolinol

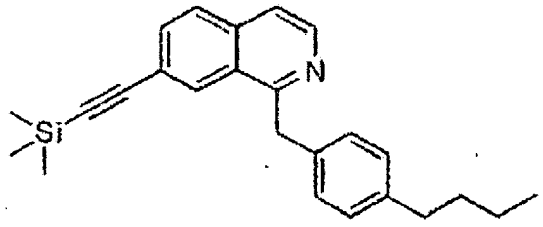


107. 1-(4-butylbenzyl)-7-isoquinolinecarbonitrile

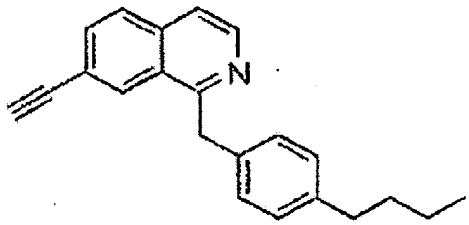


/83

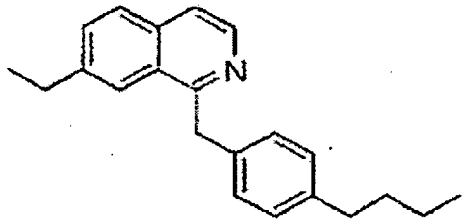
108. 1-(4-butylbenzyl)-7-[2-(1,1,1-trimethylsilyl)-1-ethynyl]isoquinoline



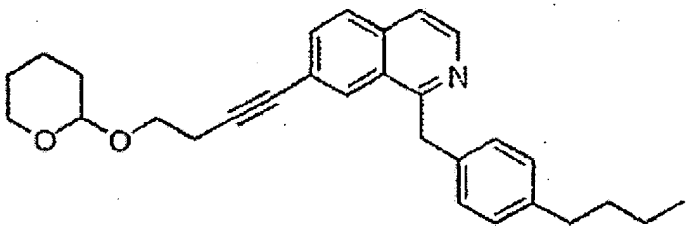
109. 1-(4-butylbenzyl)-7-(1-ethynyl)isoquinoline



110. 1-(4-butylbenzyl)-7-ethylisoquinoline

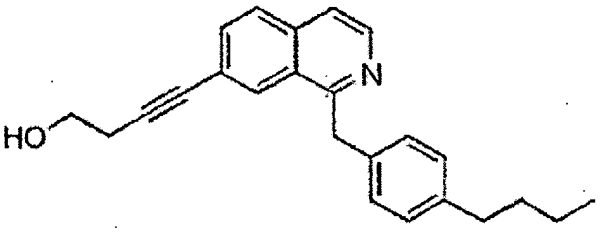


111. 1-(4-butylbenzyl)-7-[4-(tetrahydro-2H-2-pyranyloxy)-1-butynyl]isoquinoline

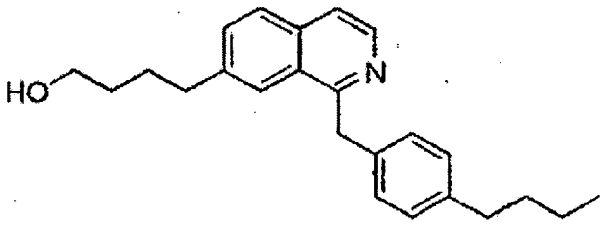


/84

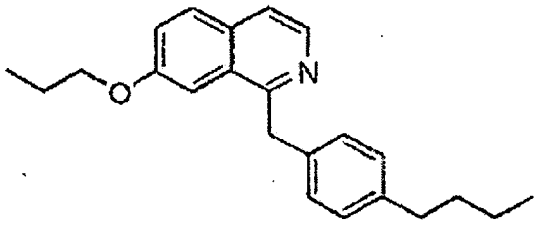
112. 4-[1-(4-butylbenzyl)-7-isoquinolyl]-3-butyn-1-ol



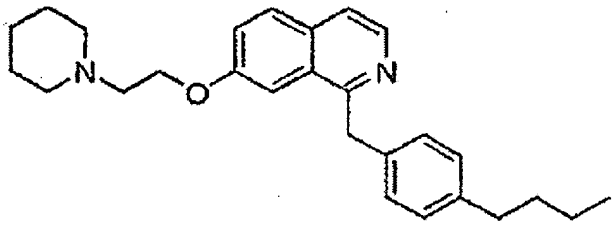
113. 4-[1-(4-butylbenzyl)-7-isoquinolyl]-1-butanol



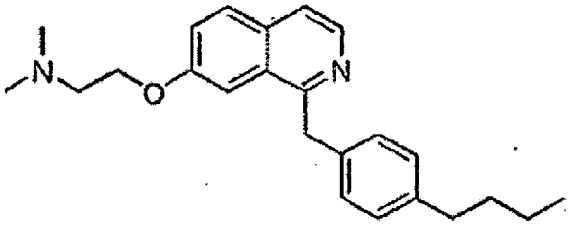
114. 1-(4-butylbenzyl)-7-propoxyisoquinoline



115. 1-(4-butylbenzyl)-7-(2-pyperidinoethoxy)isoquinoline

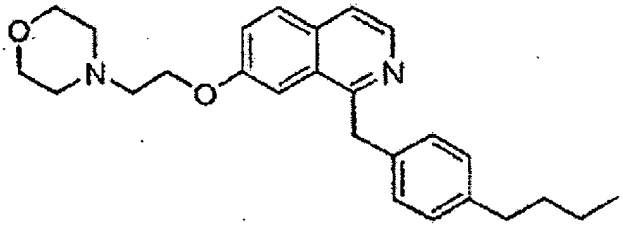


116. N-(2-{[1-(4-butylbenzyl)-7-isoquinolyl]oxy}ethyl)-N,N-dimethylamine

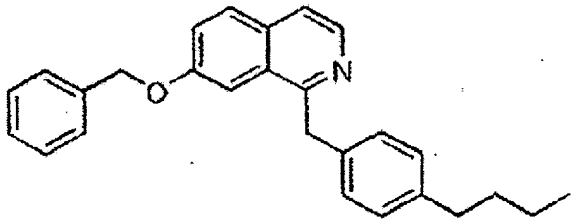


/85

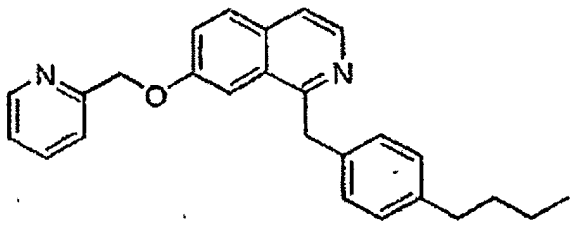
117. 1-(4-butylbenzyl)-7-isoquinolyl(2-morpholinoethyl)ether



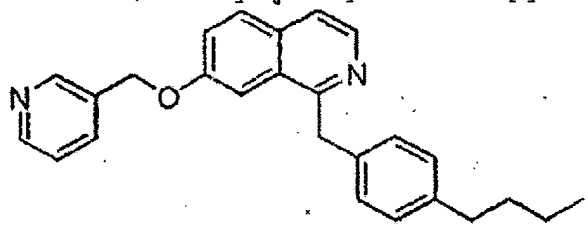
118. 7-(4-butylbenzyl)-1-(4-butylbenzyl)isoquinoline



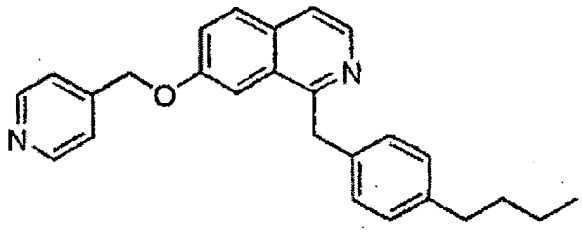
119. 1-(4-butylbenzyl)-7-(2-pyridylmethoxy)isoquinoline



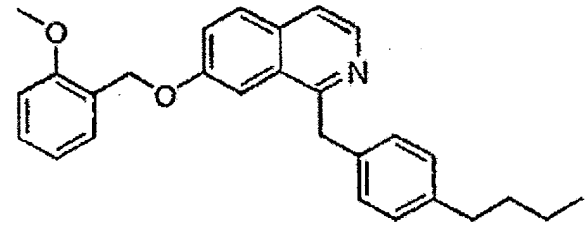
120. 1-(4-butylbenzyl)-7-(3-pyridylmethoxy)isoquinoline



121. 1-(4-butylbenzyl)-7-(4-pyridylmethoxy)isoquinoline

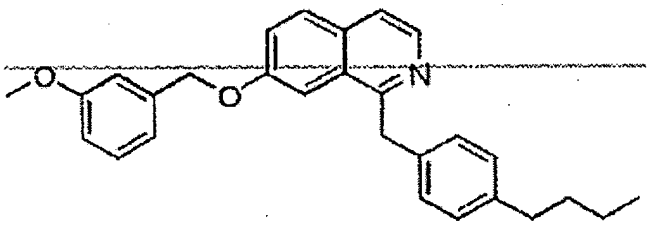


122. 1-(4-butylbenzyl)-7-[(2-methoxybenzyl)oxy]isoquinoline

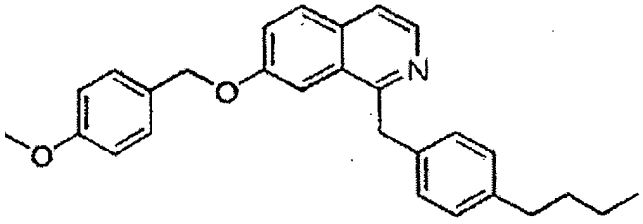


/86

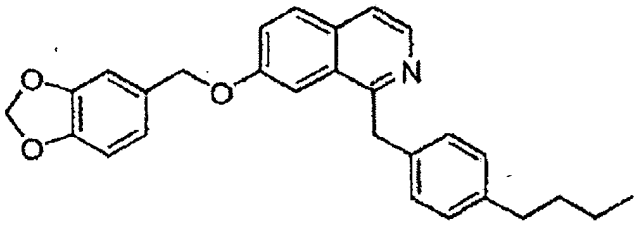
123. 1-(4-butylbenzyl)-7-[(3-methoxybenzyl)oxy]isoquinoline



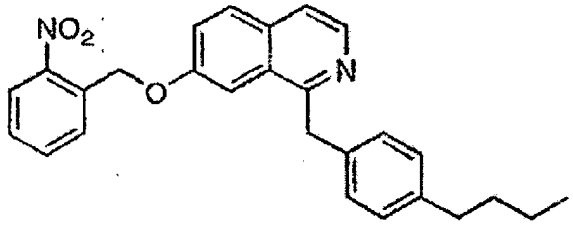
124. 1-(4-butylbenzyl)-7-[(4-methoxybenzyl)oxy]isoquinoline



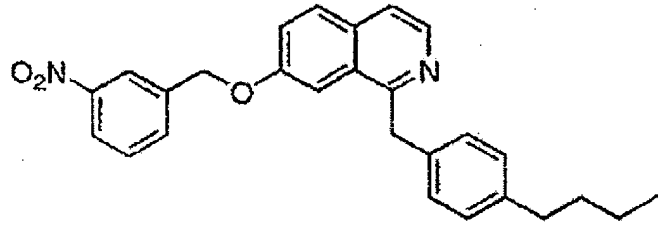
125. 7-(1,3-benzooxyol-5-tlmethoxy)-1-(4-butylbenzyl)isoquinoline



126. 1-(4-butylbenzyl)-7-[(2-nitrobenzyl)oxy]isoquinoline

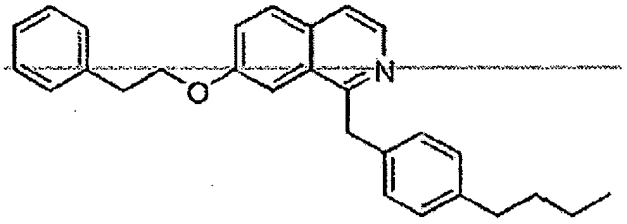


127. 1-(4-butylbenzyl)-7-[(3-nitrobenzyl)oxy]isoquinoline

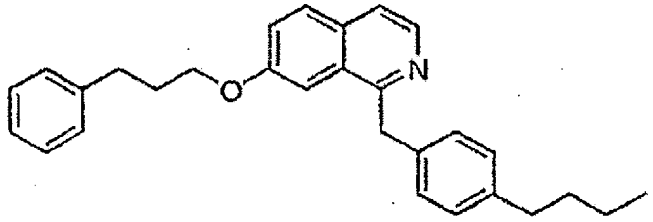


/87

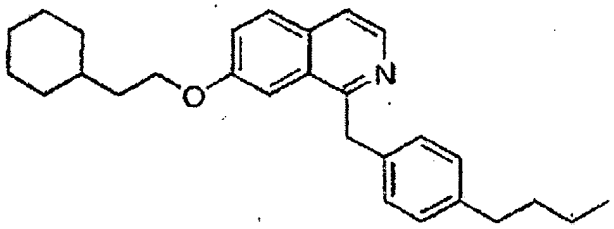
128. 1-(4-butylbenzyl)-7-(phenethyloxy)isoquinoline



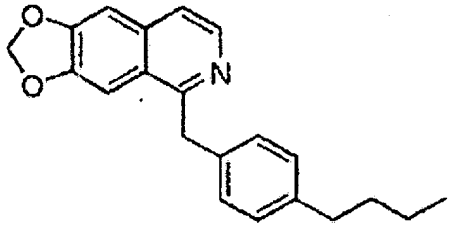
129. 1-(4-butylbenzyl)-7-(3-phenylpropoxy)isoquinoline



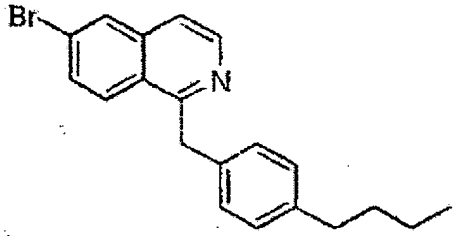
130. 1-(4-butylbenzyl)-7-(2-cyclohexylethoxy)isoquinoline



131. 5-(4-butylbenzyl)[1,3]dioxolo[4,5-g]isoquinoline

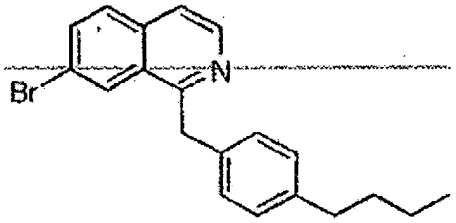


132. 6-bromo-1-(4-butylbenzyl)isoquinoline

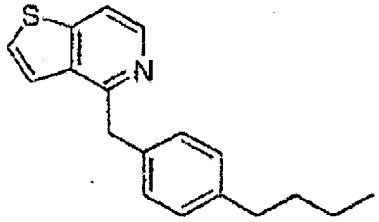


/88

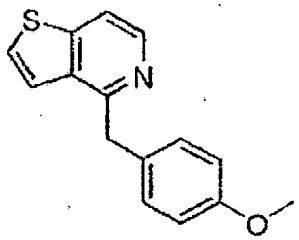
133. 7-bromo-1-(4-butylbenzyl)isoquinoline



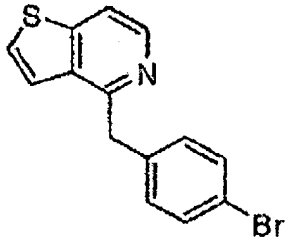
134. 4-(4-butylbenzyl)thieno[3,2-c]pyridine



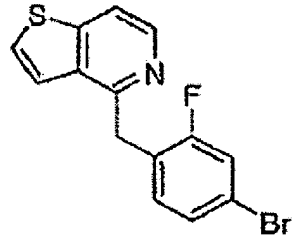
135. 4-(4-methoxybenzyl)thieno[3,2-c]pyridine



136. 4-(4-bromobenzyl)thieno[3,2-c]pyridine



137. 4-(4-bromo-2-flyorobenzyl)thieno[3,2-c]pyridine



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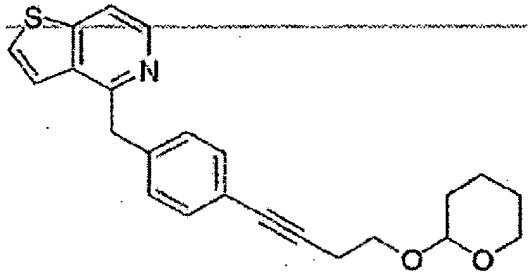
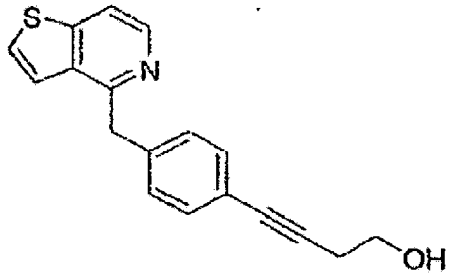
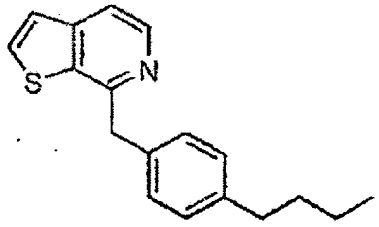
138. 4-{4-[4-(tetrahydro-2H-2-pyranyloxy)-1-butynyl]benzyl}thieno[3,2-c]pyridine



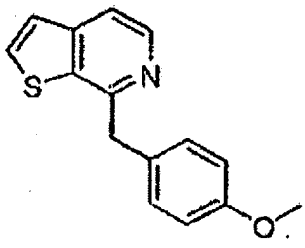
139. 4-[4-(thieno[3,2-c]pyridine-4-ylmethyl)phenyl]3-butyn-1-ol



140. 7-(4-butylbenzyl)thieno[2,3-c]pyridine



141. 7-(4-methoxybenzyl)thieno[2,3-c]pyridine



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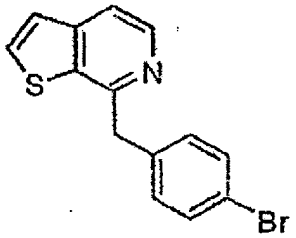
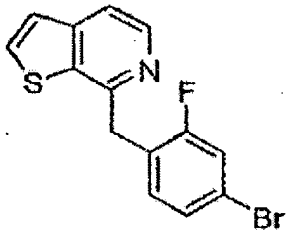
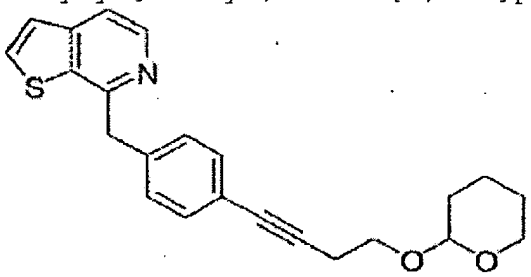
142. 7-(4-bromobenzyl)thieno[2,3-c]pyridine



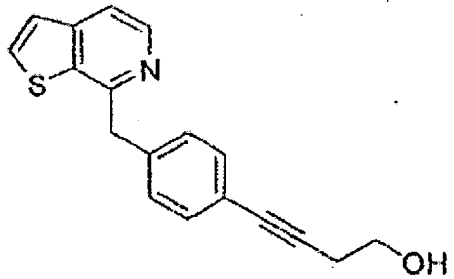
143. 7-(4-bromo-2-flyorobenzyl)thieno[2,3-c]pyridine



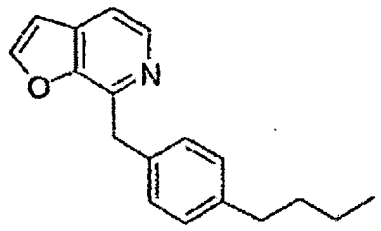
144. 7-{4-[4-(tetrahydro-2H-2-pyranyloxy)-1-butynyl]benzyl}thieno[2,3-c]pyridine



145. 4-[4-(thieno[2,3-c]pyridine-7-ylmethyl)phenyl]3-butyn-1-ol



146. 7-(4-butylbenzyl)furo[2,3-c]pyridine



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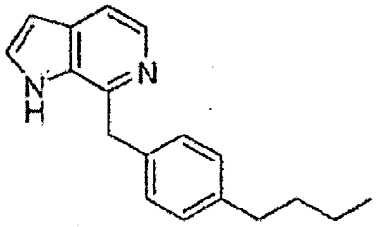
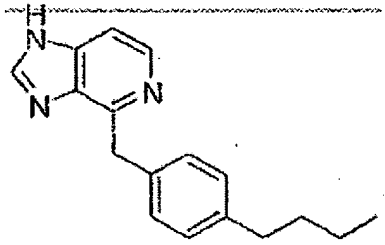
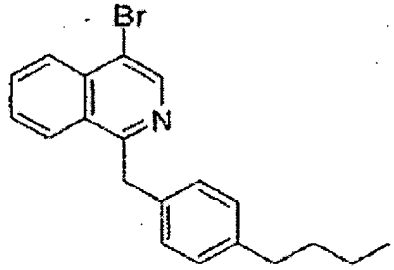
147. 7-(4-butylbenzyl)-1H-pyrrolo[2,3-c]pyridine



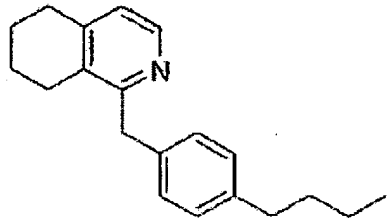
148. 4-(4-butylbenzyl)-1-imodazo[4,5-c]pyridine



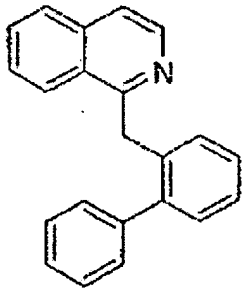
149. 4-bromo-1-(4-butylbenzyl)isoquinoline



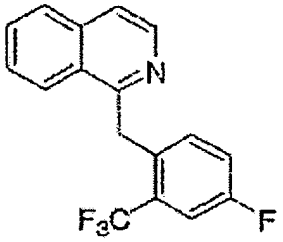
150. 1-(4-butylbenzyl)-5,6,7,8-tetrahydroisoquinoline



151. 1-[2-(phenyl)benzyl]isoquinoline



152. 1-[4-fluoro-2-(trifluoromethyl)benzyl]isoquinoline



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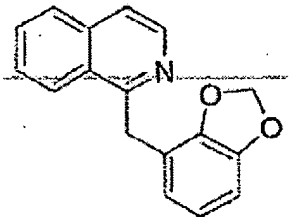
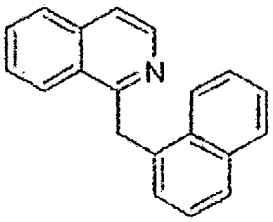
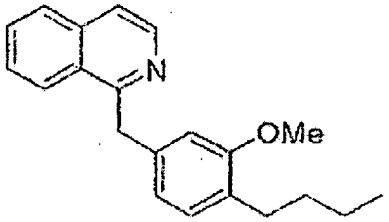
153. 1-(1,3-benzodioxoyl-4-ylmethyl)isoquinoline



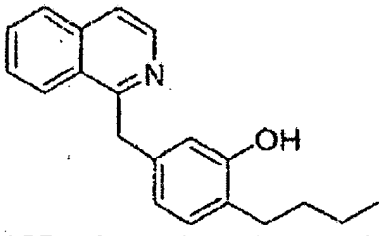
154. 1-(1-naphthylmethyl)isoquinoline



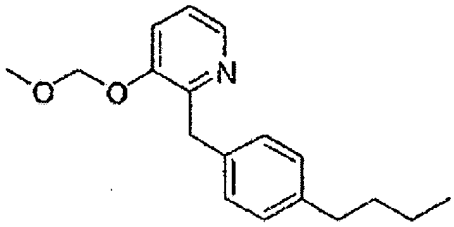
155. 1-(4-butyl-3-methoxybenzyl)isoquinoline



156. 2-butyl-5-(1-isoquinolylmethyl)phenol

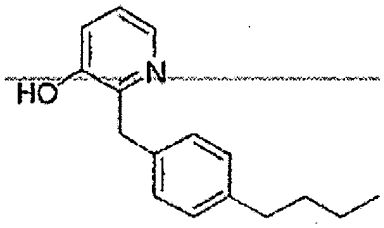


157. 2-(4-butylbenzyl)-3-(methoxymethoxy)pyridine

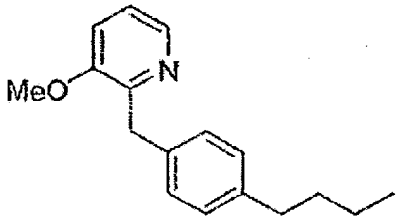


/93

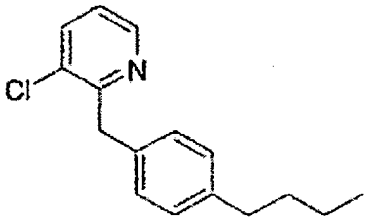
158. 2-(4-butylbenzyl)-3-pyridinol



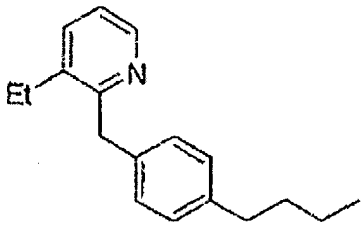
159. 2-(4-butylbenzyl)-3-methoxypyridine



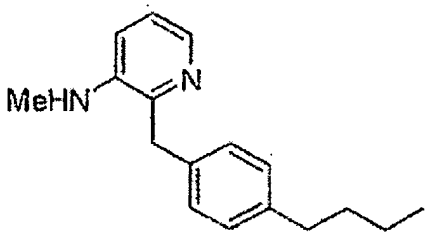
160. 2-(4-butylbenzyl)-3-chloropyridine



161. 2-(4-butylbenzyl)-3-ethylpyridine

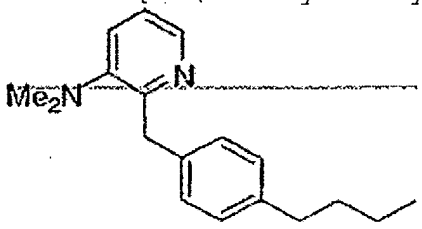


162. N-[2-(4-butylbenzyl)-3-pyridyl]-N-methylamine

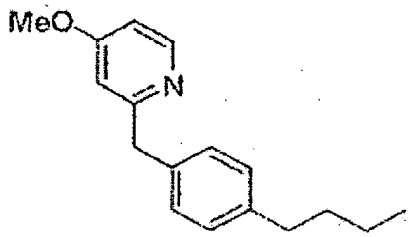


/94

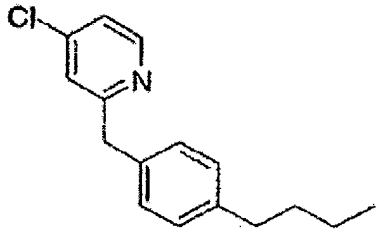
163. N-[2-(4-butylbenzyl)-3-pyridyl]-N,N-dimethylamine



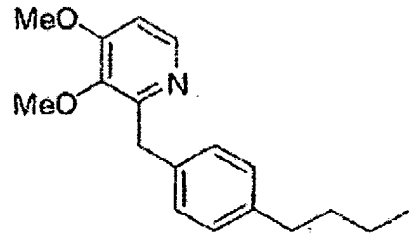
164. 2-(4-butylbenzyl)-4-methoxypyridine



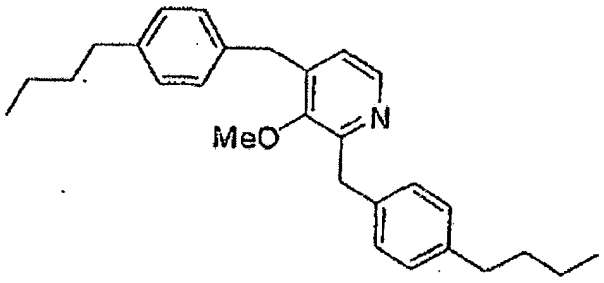
165. 2-(4-butylbenzyl)-4-chloropyridine



166. 2-(4-butylbenzyl)-3,4-dimethoxypyridine

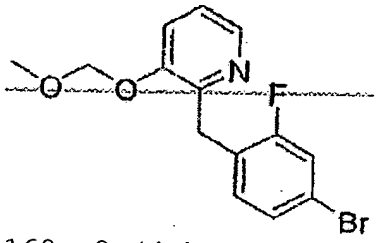


167. 2,4-di(4-butylbenzyl)-3-methoxypyridine

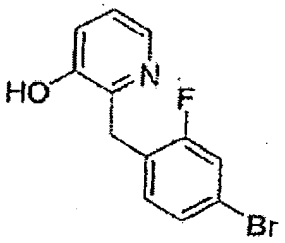


/95

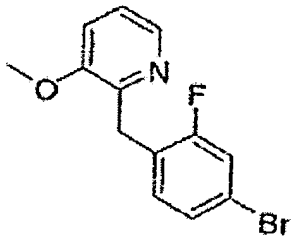
168. 2-(4-bromo-2-fluorobenzyl)-3-(methoxymethoxy)pyridine



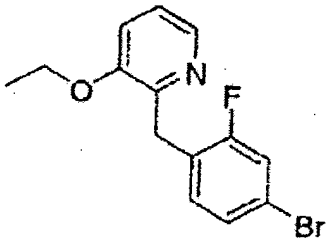
169. 2-(4-bromo-2-fluorobenzyl)-3-pyridinol



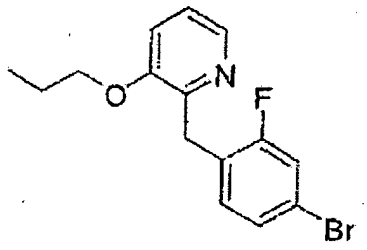
170. 2-(4-bromo-2-fluorobenzyl)-3-methoxypyridine



171. 2-(4-bromo-2-fluorobenzyl)-3-ethoxypyridine

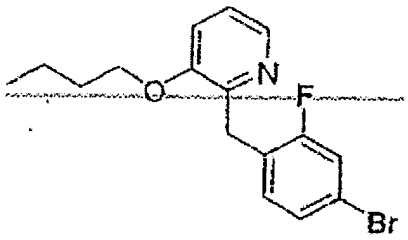


172. 2-(4-bromo-2-fluorobenzyl)-3-propoxypyridine

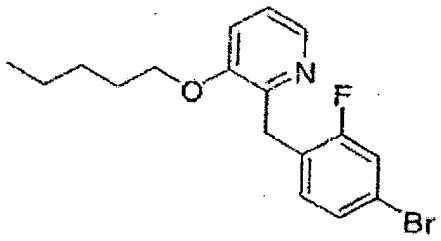


/96

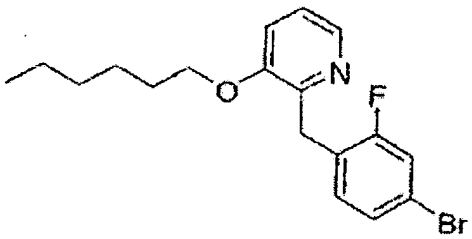
173. 2-(4-bromo-2-fluorobenzyl)-3-butoxypyridine



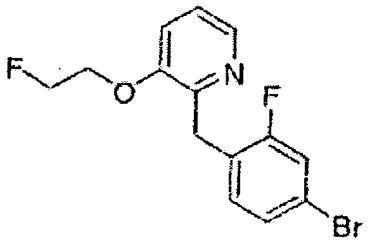
174. 2-(4-bromo-2-fluorobenzyl)-3-(pentyloxy)pyridine



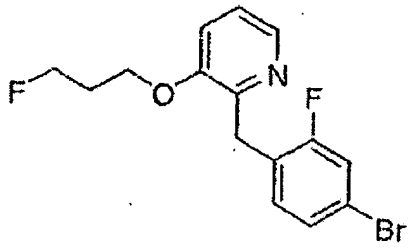
175. 2-(4-bromo-2-fluorobenzyl)-3-(hexyloxy)pyridine



176. 2-(4-bromo-2-fluorobenzyl)-3-(fluoroethoxy)pyridine

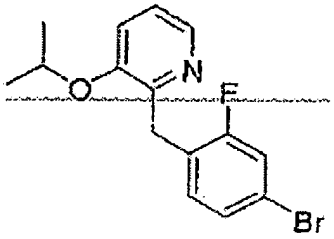


177. 2-(4-bromo-2-fluorobenzyl)-3-(fluoropropoxy)pyridine

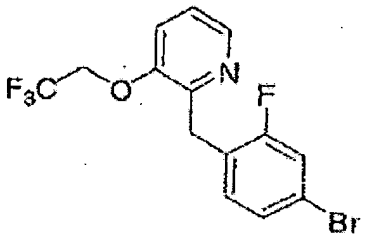


/97

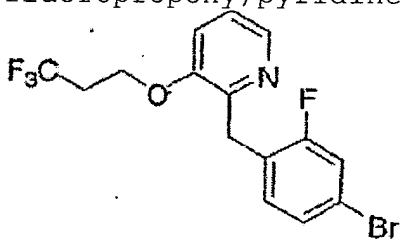
178. 2-(4-bromo-2-fluorobenzyl)-3-isopropoxypyridine



179. 2-(4-bromo-2-fluorobenzyl)-3-(2,2,2-fluoroethoxy)pyridine



180. 2-(4-bromo-2-fluorobenzyl)-3-(3,3,3-fluoropropoxy)pyridine



/98

Potential Industrial Applications

Thanks to the present invention, the compounds which inhibit the process of transporting a GPI-anchored protein to a fungal cell wall can be screened by a simple binding assay.

/99

What is Claimed is:

1. A screening method for compound having an antifungal activity, wherein the method comprises the steps of:

(1) A process wherein a protein encoded by a DNA described in any of items (a) to (e), a test sample and a labeled compound having a binding activity to the protein are contacted;

(a) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 2, 4, 6, 28, 40 or 59;

(b) a DNA comprising the nucleotide sequence of SEQ ID NO: 1, 3, 5, 27, 39, 41, 54 or 58;

(c) a DNA hybridizing to the DNA comprising the nucleotide sequence of SEQ ID NO: 1, 3, 5, 27, 39, 41, 54 or 58 under stringent conditions;

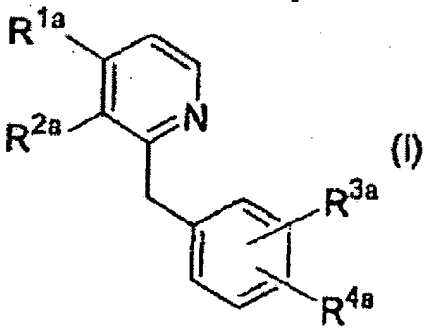
(d) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 2, 4, 6, 28, 40 or 59, wherein one or more amino acids have been added, deleted, substituted and/or inserted; and

(e) a DNA amplified using primers (SEQ ID NO: 29 and 31 or SEQ ID NO: 29 and 30).

(2) A process wherein a labeled compound which binds to the protein is detected; and

(3) A process wherein a test sample which reduces the labeled compound which binds to the protein is selected.

2. The method in accordance with Claim 1, wherein the labeled compound having a binding activity to the protein is represented by the following formula (I):

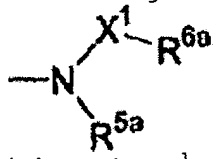


(I)

/100

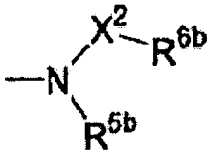
[wherein R^{1a} and R^{2a} identically or independently represent a hydrogen atom, halogen atom, a hydroxyl group, a nitro

group, a cyano group, a trifluoromethyl group, a trifluoromethoxy group, a C_{1-6} alkyl group which may be substituted, a C_{2-6} alkenyl group, a C_{2-6} alkynyl group, a C_{1-6} alkoxy group or a group that can be represented by the following formula:



(wherein X^1 represents a single bond, a carbonyl group or a group that can be represented by $-S(O)_2-$; R^{5a} and R^{6a} may be the same or different and each represents a hydrogen atom or a C_{1-6} alkyl group which may be substituted).
Alternatively, R^{1a} and R^{2a} may together form a condensed ring selected from a group of a benzene ring which may be substituted, a pyridine ring which may be substituted, a pyrrole ring which may be substituted, a thiophene ring which may be substituted, a furan ring which may be substituted, a pyridazine ring which may be substituted, a pyrimidine ring which may be substituted, a pyrazine ring which may be substituted, an imidazole ring which may be substituted, an oxazole ring which may be substituted, a thiazole ring which may be substituted, a pyrazole ring which may be substituted, an isooxazole ring which may be substituted, an isothiazole ring which may be substituted, a cyclohexane ring which may be substituted and a cyclopentane ring which may be substituted;
R^{3a} and R^{4a} may be the same or different and each represents a hydrogen atom, a halogen atom, a hydroxyl group, a nitro group, a cyano group, a carboxyl group, a formyl group, a hydroxyimino group, a trifluoromethyl group, a trifluoromethoxy group, a C_{1-6} alkyl group, a C_{1-6} alkoxy group, a C_{2-6} alkenyl group, a C_{2-6} alkynyl group, a group that can be represented by the formula: $-C(O)NR^{7a}R^{7b}$ (wherein R^{7a} and R^{7b} may be the same or different and each represents a hydrogen atom or a C_{1-6} alkyl group), the formula: $-CO_2R^{7a}$ (wherein R^{7a} is the same as above), the formula: $-S(O)_nR^{7a}$ (wherein n donates an integer of 0 to 2. R^{7a} is the same as above), the formula: $-S(O)_2NR^{7a}R^{7b}$ (wherein R^{7a} and R^{7b} are the same as above) and the following formula:

/101



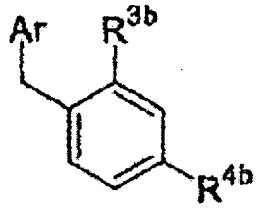
(wherein X^2 represents a single bond, a carbonyl group or a group that can be represented by formula: $-S(O)_2-$; R^{5a} and R^{6a} may be the same or different and each represents a hydrogen atom, a C_{1-6} alkyl group which may be substituted or a C_{6-14} aryl group which may be substituted), or a group that can be represented by the following formula:

$-Z^1-Z^2$

(wherein Z^1 represents a single bond, an oxygen atom, a vinylene group or an ethynylene group; Z^2 represents a single bond or a C_{1-6} alkyl group which may be substituted by 0 to 4 substituents). R^{3a} and R^{4a} may together represent a methylenedioxy group or a 1,2-ethylenedioxy group, or may together form a condensed ring selected from a group of a benzene ring which may be substituted, a pyridine ring which may be substituted, a pyrrole ring which may be substituted, a thiophene ring which may be substituted, a furan ring which may be substituted, a pyridazine ring which may be substituted, a pyrimidine ring which may be substituted, a pyrazine ring which may be substituted, an imidazole ring which may be substituted, an oxazole ring which may be substituted, a thiazole ring which may be substituted, a pyrazole ring which may be substituted, an isooxazole ring which may be substituted, an isothiazole ring which may be substituted, a cyclohexane ring which may be substituted and a cyclopentane ring which may be substituted unless R^{1a} and R^{2a} identically represent a hydrogen atom].

/102

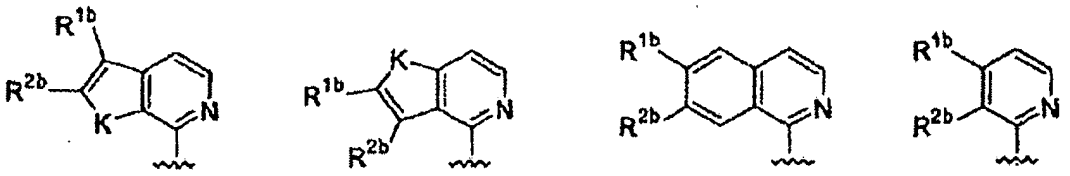
3. The method in accordance with Claim 1, wherein the labeled compound having a binding activity to the protein is represented by the following formula (I):



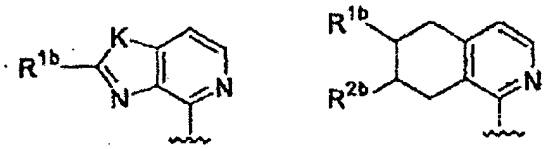
(II)

[wherein Ar represents a substituent selected from a group of (IIIa) to (IIIf):

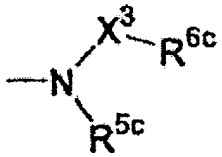
(IIIa) (IIIb) (IIIc) (IIId)



(IIIe) (IIIf)



(wherein K represents a sulfur atom, an oxygen atom or a group that can be represented by the formula: -NH-; R^{1b} and R^{2b} may be the same or different and each represents a hydrogen atom, a halogen atom, a hydroxyl group, a nitro group, a cyano group, a trifluoromethyl group, a trifluoromethoxy group, a C_{1-6} alkyl group which may be substituted, a C_{1-6} alkoxy group which may be substituted, a group that can be represented by the following formula:



(wherein X^3 represents a single bond, a carbonyl group or a group that can be represented by formula: $-S(O)_2-$;

/103

R^{5c} and R^{6c} may be the same or different and each represents a hydrogen atom or a C_{1-6} alkyl group which may be substituted), or a group that can be represented by formula: $-X^4-R^{8a}$ (wherein X^4 represents a single bond, an oxygen atom or a sulfur atom; R^{8a} represents a C_{1-6} alkyl group, a C_{2-6} alkenyl group, a C_{2-6} alkynyl group, a C_{3-8} cycloalkyl group or a C_{3-8} cycloalkenyl group. Further, R^{1b} and R^{2b} may together form a methylenedioxy group or a 1,2-ethylenedioxy group);
R^{3b} and R^{4b} may be the same or different and each represents a hydrogen atom, a halogen atom, a hydroxyl group, a nitro group, a cyano group, a carboxyl group, a formyl group, a hydroxyimino group, a trifluoromethyl group, a trifluoromethoxy group, a C_{1-6} alkyl group, a C_{1-6} alkoxy

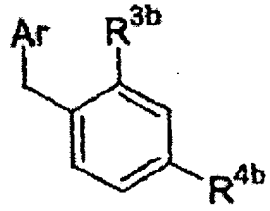
group, a C_{2-6} alkenyl group, a C_{2-6} alkynyl group or a group that can be represented by the following formula:

$$—Z^{1b}—Z^{2b}$$

(wherein Z^{1b} represents a single bond, a vinylene group or an ethynylene group; Z^{2b} represents a single bond or a C_{1-6} alkyl group which may be substituted by 0 to 4 substituents). unless (1) Ar can be represented by the above formula (IIId) in which both R^{1b} and R^{2b} represent a hydrogen atom, (2) at least either R^{3b} or R^{4b} represents a hydrogen atom and the other represents a hydrogen atom, a methoxy group, a hydroxyl group, a methyl group, a benzyloxy group or a halogen atom, and Ar can be represented by the above formula (IIIc) in which both R^{1b} and R^{2b} represent a hydrogen atom or a methoxy group, (3) at least either R^{3b} or R^{4b} represents a hydrogen atom and the other represents a hydrogen atom, a hydroxyl group, a methoxy group or a benzyloxy group, and Ar can be represented by the formula (IIIc) in which both R^{1b} and R^{2b} represent a hydroxyl group or a benzyloxy group, or (4) Ar can be represented by the above formula (IIId) in which R^{1b} represents a hydrogen atom and R^{2b} represents a formyl group, hydroxymethyl group or methoxycarbonyl group],

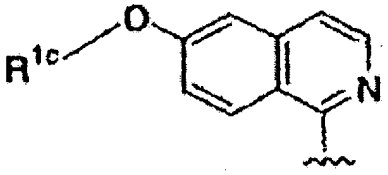
/104

4. The method in accordance with Claim 1, wherein the labeled compound having a binding activity to the protein is represented by the following formula (I):



(II)

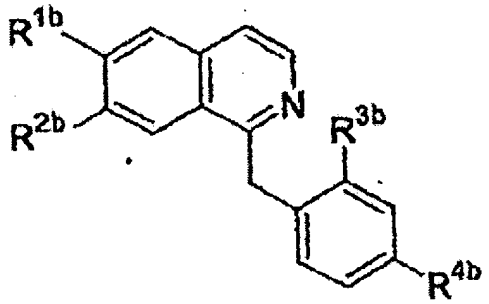
[wherein Ar can be represented by the following formula:



(IIIg)

(wherein R^{1c} represents a hydrogen atom, a C_{1-6} alkyl group which may be substituted or a benzyl group) unless R^{3b} represents a hydrogen atom].

5. The method in accordance with Claim 1, wherein the labeled compound having a binding activity to the protein is represented by the following formula (IIIc2):



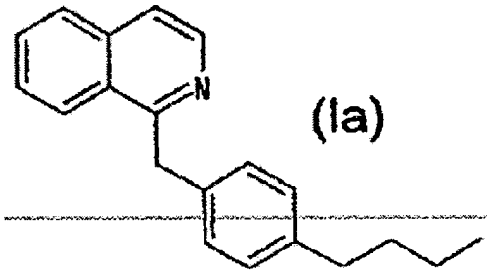
(IIIc2)

[wherein R^{1b} and R^{2b} are the same as above. unless (1) R^{1b} can be represented by the formula: R^{1c}-O- (wherein R^{1c} is the same as above), R^{2b} represents a hydrogen atom and R^{3b} represents a hydrogen atom, (2) at least either R^{3b} or R^{4b} represents a hydrogen atom and the other represents a hydrogen atom, a methoxy group, a hydroxyl group, a methyl group, a benzyloxy group or a halogen atom, and both R^{1b} and R^{2b} represent a hydrogen atom or a methoxy atom, (3) at least either R^{3b} or R^{4b} represents a hydrogen atom and the other represents a hydrogen atom, a hydroxyl group, a

/105

methoxy group or a benzyloxy group, and both R^{1b} and R^{2b} represent a hydroxyl group or a benzyloxy group].

6. The method in accordance with Claim 1, wherein the labeled compound having a binding activity to the protein is represented by the following formula (Ia):



(Ia)

7. The method in accordance with any of Claims 1 through 6, comprising a process (4) which determines whether the selected test sample inhibits the process of transporting a GPI-anchored protein to a fungal cell wall or whether the test sample inhibits the expression of a GPI-anchored protein on a fungal cell surface.

FIG. 1

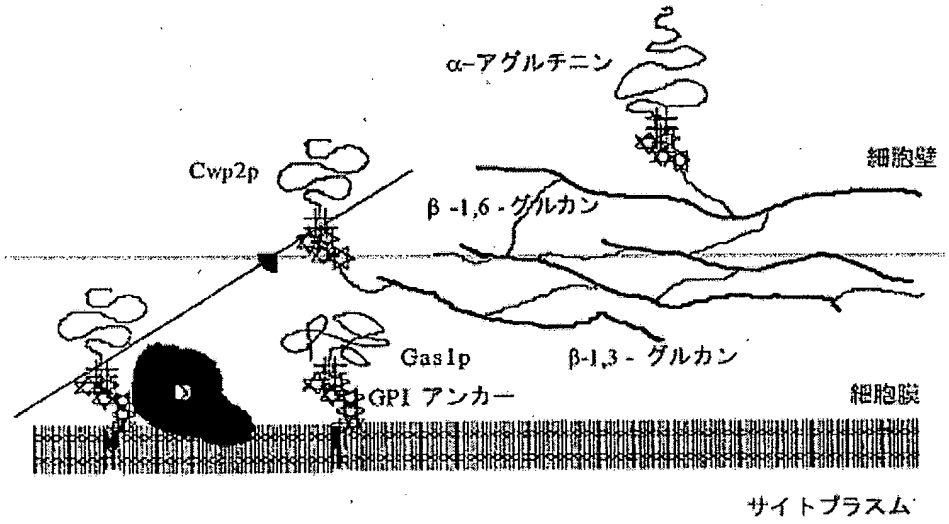

α-アグルチニン
細胞壁
Cwp2p
β -1,6 - グルカン
Gas1p
β-1,3 - グルカン
GPI アンカー
細胞膜
サイトプラスム

FIG. 2

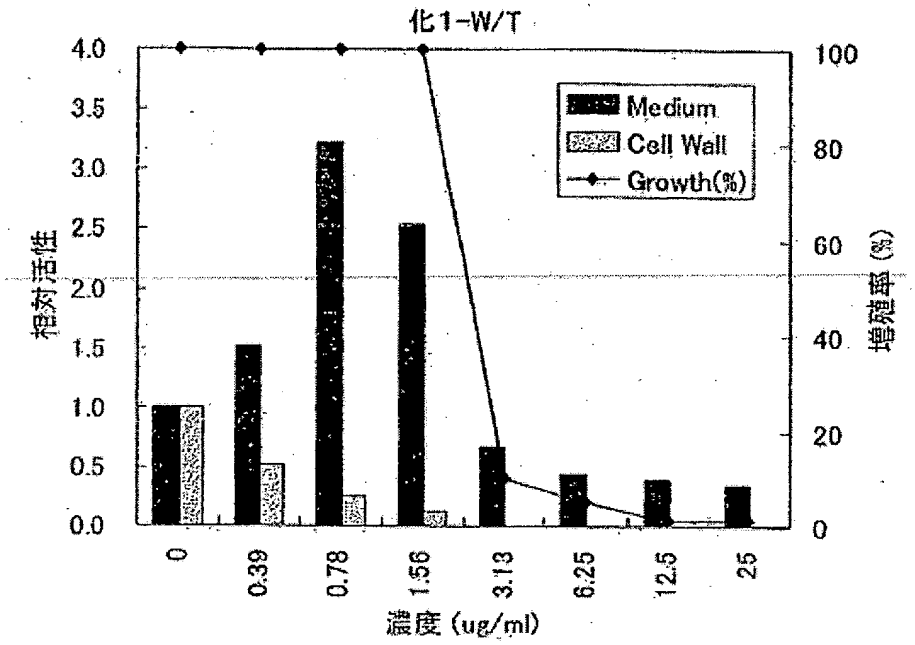

化1-W/T
Medium
Cell Wall
Growth(%)
相対活性
増殖率 (%)
濃度 (ug/ml)
0
0.39
0.78
1.56
3.13
6.25
12.5
25
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
0
20
40
60
80
100

FIG. 3

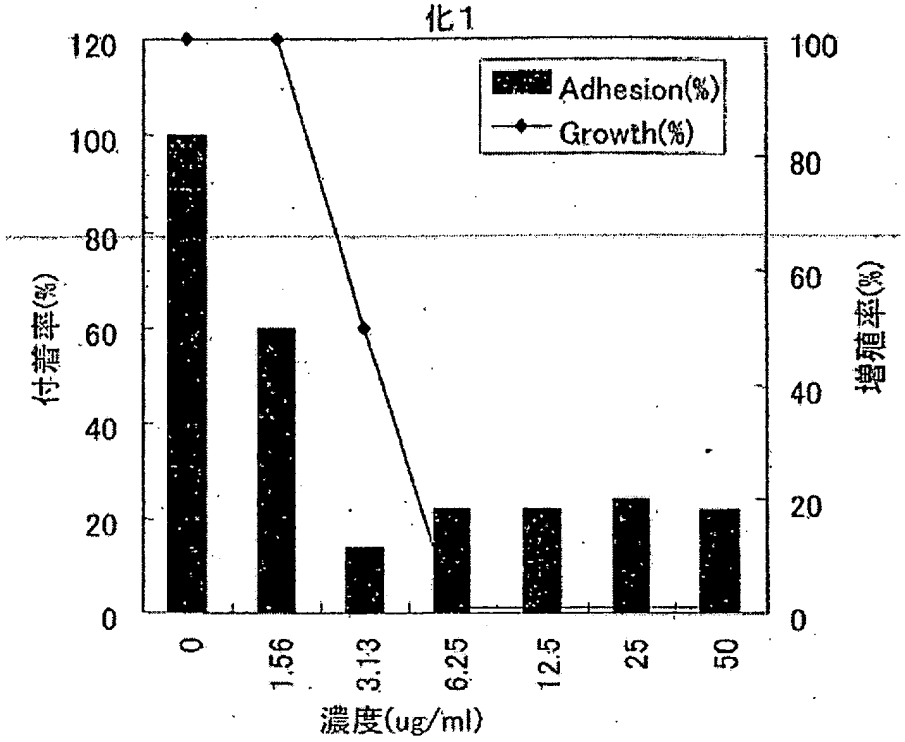

化1
Adhesion(%)
Growth(%)
付着率(%)
増殖率(%)
濃度(ug/ml)
0
1.56
3.13
6.25
12.5
25
50
120
100
80
60
40
20
0

FIG. 4

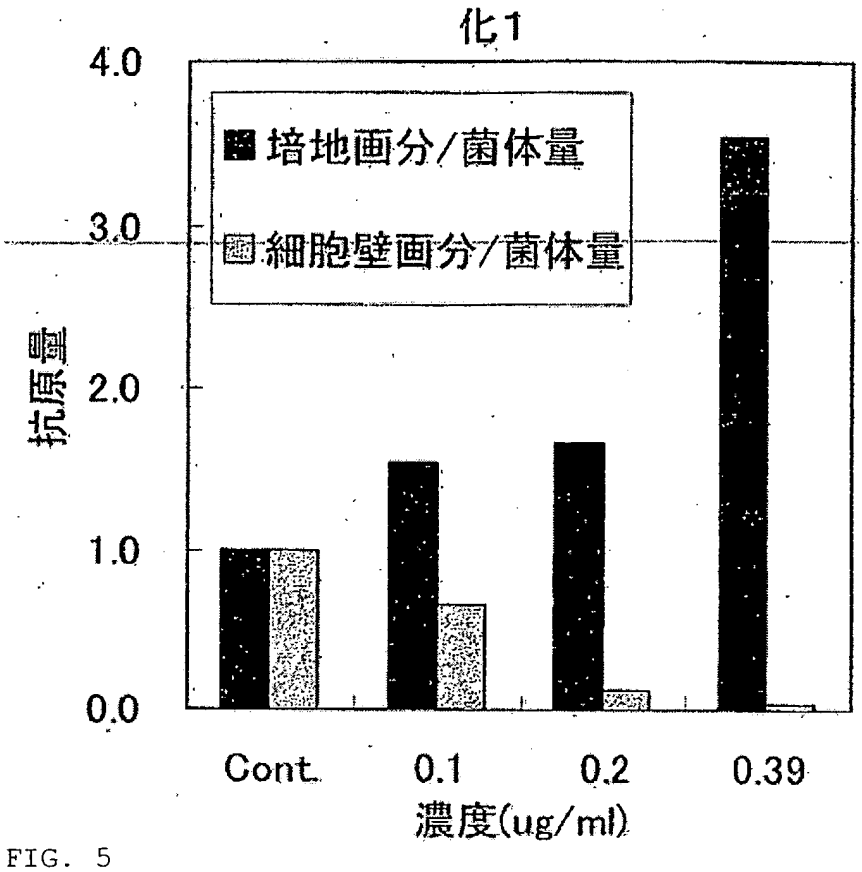

化1
4.0
3.0
2.0
1.0
0.0
抗原量
培地画分/菌体量
細胞壁画分/菌体量
Cont.
0.1
0.2
0.39
濃度(ug/ml)

FIG. 5

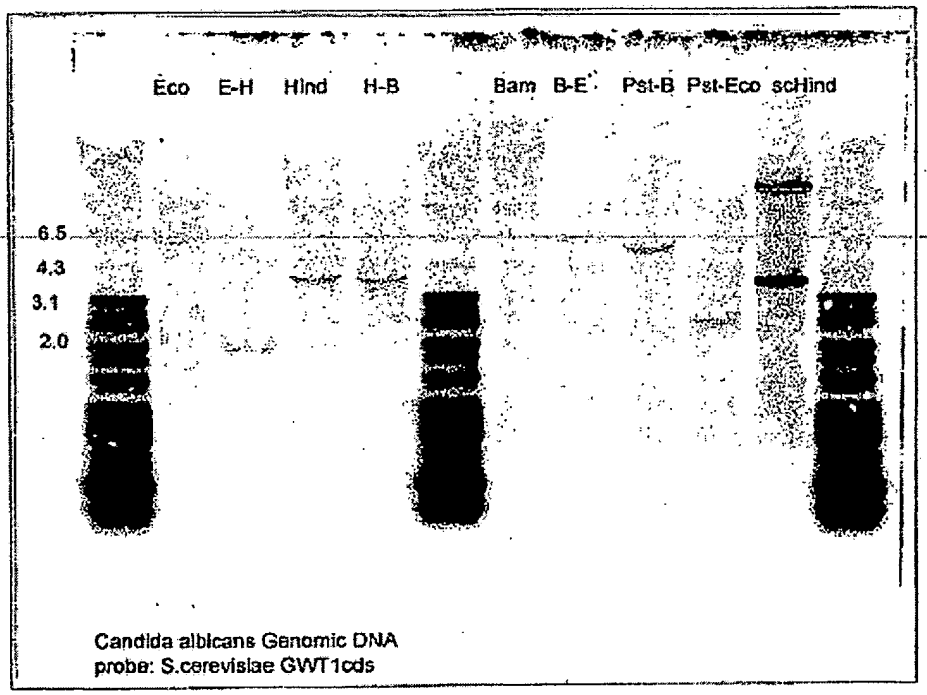

Eco
E-H
Hind
H-B
Bam
B-E
Pst-B
Pst-Eco
scHind
6.5
4.3
3.1
2.0
Candida albicans Genomic DNA
probe: S.cerevisiae GWT1cds

FIG. 6

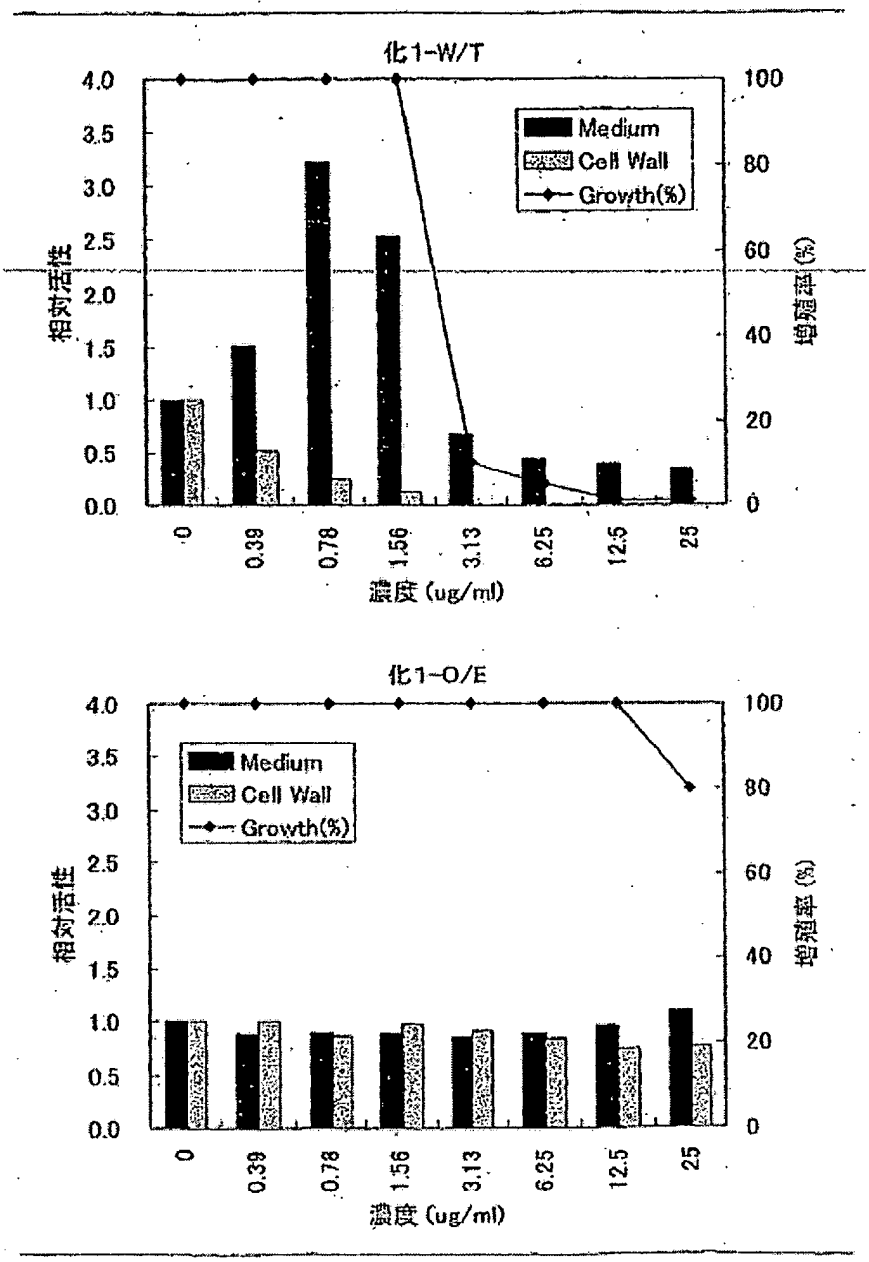

化1-W/T
Medium
Cell Wall
Growth(%)
相対活性
増殖率(%)
濃度(ug/ml)
0
0.39
0.78
1.56
3.13
6.25
12.5
25
化1-O/E
Medium
Cell Wall
Growth(%)
相対活性
増殖率(%)
濃度(ug/ml)

FIG. 7

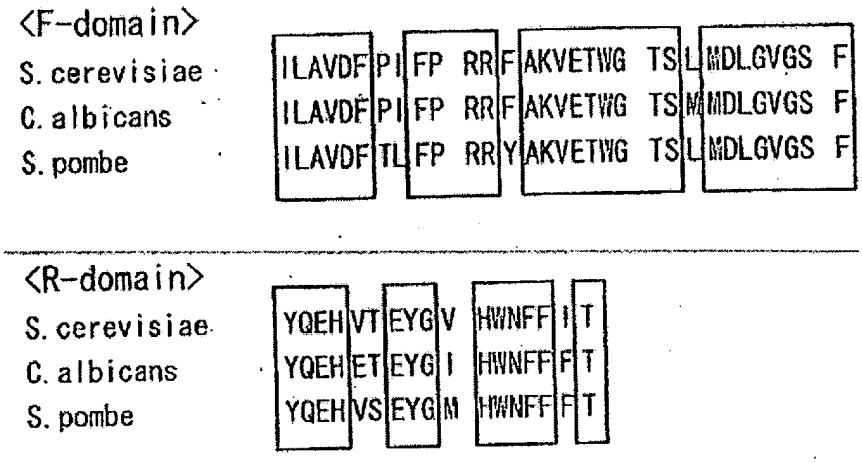

<F-domain>
S. cerevisiae
C. albicans
S. pombe
ILAVDF PI FP RR F AKVETWG TS L MDLGVGS F
ILAVDF PI FP RR F AKVETWG TS M MDLGVGS F
ILAVDF TL FP RR Y AKVETWG TS L MDLGVGS F
<R-domain>
S. cerevisiae
C. albicans
S. pombe
YQEH VT EYG V HWNFF I T
YQEH ET EYG I HWNFF F T
YQEH VS EYG M HWNFF F T

FIG. 8

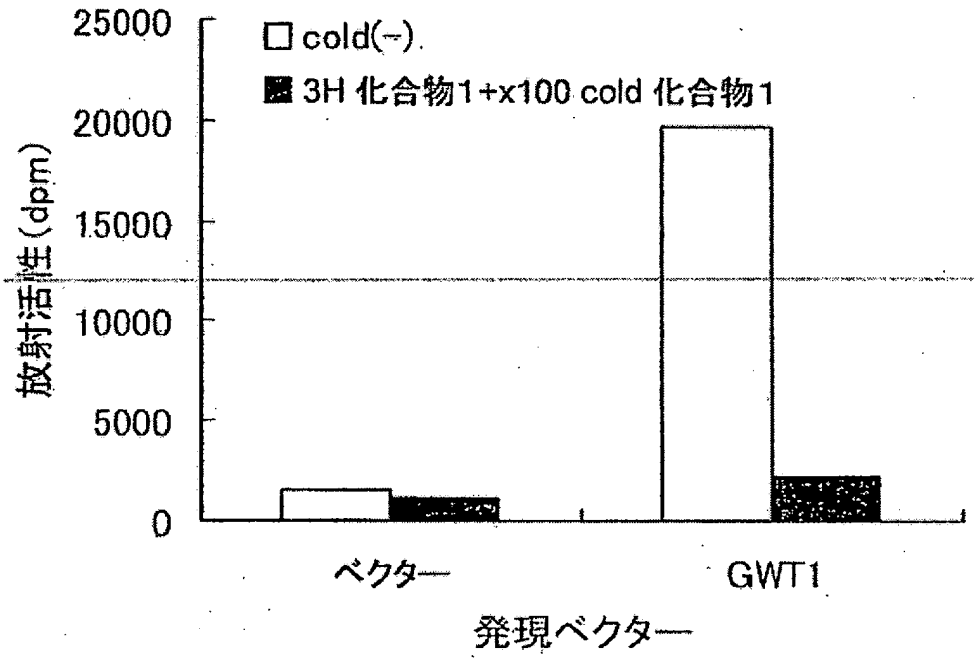

25000
20000
15000
10000
5000
0
放射活性(dpm)
cold(-)
3H 化合物1+x100 cold 化合物1
ベクター
GWT1
発現ベクター

FIG. 9

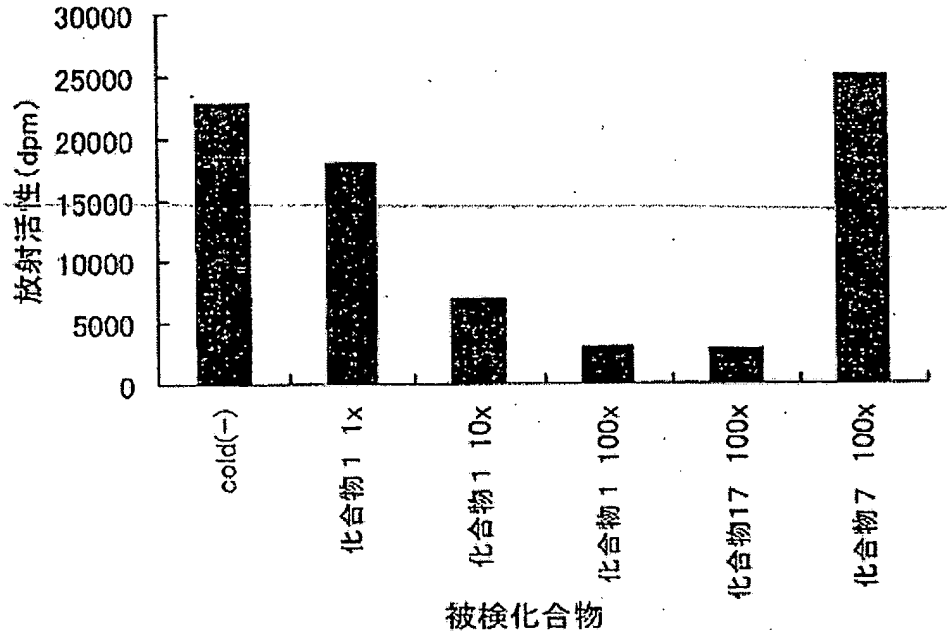
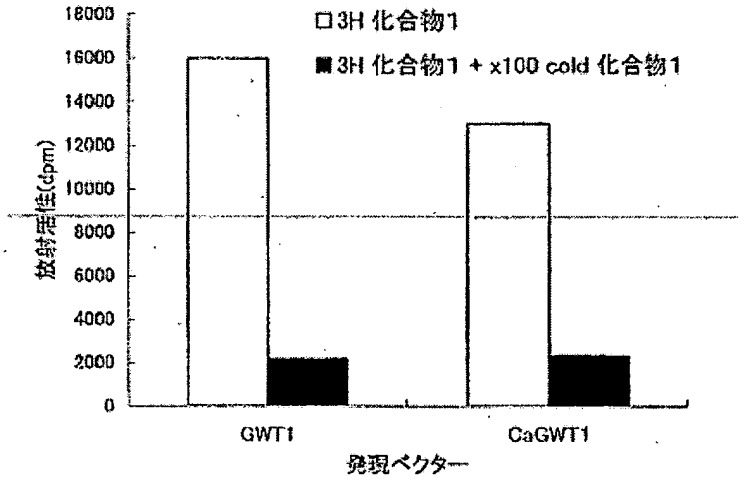

30000
25000
20000
15000
10000
5000
0
放射活性(dpm)
cold(−)
化合物1 1x
化合物1 10x
化合物1 100x
化合物17 100x
化合物7 100x
被検化合物

FIG. 10


□3H 化合物1
■3H 化合物1 + x100 cold 化合物1
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
放射活性(dpm)
GWT1
CaGWT1
発現ベクター